A STUDY OF THE EPIDEMIOLOGY, PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF GUILLAIN BARRÉ SYNDROME ASSOCIATED CAMPYLOBACTERIOSIS

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David

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LIST OF ABBREVIATIONS

A	adenine
AAB	Advanced American Biotechnology
AFLP	amplified fragment length polymorphism
AIDP	acute demyelinating polyneuropathy
AIDS	acquired immuno-defiency syndrome
AMAN	acute motor axonal neuropathy
AMSAN	acute motor-sensory axonal neuropathy
anti-	antibodies directed against
APS	ammonium persulphate
APW	alkaline peptone water
ATCC	American type culture collection
b.p.	base pairs
BZ	Bruce-Zochowsky medium
C	cytosine
CAT	cefoperazone amphotericin teicoplanin
CCDA	charcoal cefoperazone deoxycolate agar
CSF	cerebrospinal fluid
CDI	Communicable Disease Intelligence
CDS	calibrated dichotomous sensitivity
CEB	*Campylobacter* enrichment broth
CFU	colony forming unit
CNW	catalase negative/weak
CO_2	carbon dioxide
CSM	charcoal based selective media
CSPD	Disodium 3 – (4-methoxyspiro {1,2 – dioxetane – 3,2' (5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate

Ctx	*Cholera* toxin
CT	campy-thio broth
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
dNTP	deoxynucleoside triphosphate
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ESP	EDTA, Sarcosine, Proteinase K solution
G	guanine
GBS	Guillain-Barré syndrome
GLC	gas-liquid chromatography
GyrB	Gyrase B gene
HBA	horse blood agar
HCl	hydrochloric acid
H_2SO_4	sulphuric acid
Ig	immunoglobulin
IPTG	isopropyl β-D-Thiogalactopyranoside
kb	kilobases
LB	Luria-Bertani
LOS	lipooligosaccharides
LMP	low melting point agarose
LPS	lipopolysaccharides
m	milli (10^{-3})

M	molar (mol/L)
MDU	Melbourne Diagnostic Unit
MgCl	magnesium chloride
MFS	Miller Fisher Syndrome
MLST	multi locus sequence typing
MWM	molecular weight marker
n	nano (10^{-9})
N_2	nitrogen
NaCl	sodium chloride
NARTC	nalidixic acid resistant thermophilic *Campylobacter*
NAD	Nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NCTC	National Culture Type Collection
NNC	nitrate negative *Campylobacter*
NNDSS	National Notifiable Disease Surveillance Database System
n.t.	nucleotides
O_2	oxygen
OD	optical density
OPD	o-Phenylenediamine Dihydrochloride
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PIV	Petts IV solution
RAPD	random amplified polymorphic DNA
RHH	Royal Hobart Hospital
rRNA	ribosomal ribonucleic acid
RFLP	random fragment length polymorphism

RT	room temperature
SDS	sodium lauryl sulphate
SMTM	semi-solid motility media
SKM	skirrow medium
SSC	sodium chloride, sodium citrate solution
SSM	semi-solid motility media
ST	sequence type
T	thymine
TAE	tris-acetate EDTA buffer
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris, Boric-Acid, EDTA buffer
TEMED	tetramethylenediamine
TE1	Tris.HCl, EDTA buffer
TPHMD	Tasmanian Public Health Morbidity Database
Tris base	(Hydroxymethyl) Aminomethane
Tris.HCl	(Hydroxymethyl) Aminomethane Hydrochloride
L	litres
UPGMA	unweighted-pair group method with arithmetic averages
UPTC	urea positive thermophilic *Campylobacter*
UV	ultraviolet
V	volts
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
°C	degress celsius
μ	micro (10^{-6})

FOREWORD

Guillain-Barré syndrome (GBS) is a neurological disease characterised by ascending paralysis that can lead to respiratory muscle compromise and death. Although the exact trigger of GBS is unknown, case control studies have shown that it often follows an acute infectious illness. In recent years, serological and cultural studies have suggested that *Campylobacter jejuni* is the infectious agent most commonly associated with the development of GBS. Culture confirmation of *C. jejuni* infections has been achieved in up to 50% of GBS patients, in spite of the fact that many GBS patients with antecedent *Campylobacter* infection are likely to have already cleared their stools of the organism by the time neurological symptoms begin. *Campylobacter* infection is now classified as the most common cause of bacterial food poisoning in the Western world. In Tasmania, over 400 cases are notified each year. Given this prevalence it is not clear why only a small number of patients with *C. jejuni* enteritis develop GBS whilst the majority do not. Two possibilities exist; the first being that susceptibility is determined by host-specific factors; the second is that bacterial strain-specific factors determine whether patients develop GBS. The aim of this investigation is to determine the importance of the second factor. The specific objectives being to define specific bacterial markers that might be used to determine those strains of *Campylobacter* associated with the development of GBS.

The way in which infection with *C. jejuni* leads to GBS is unknown, however, there is evidence suggesting that Gm1 ganglioside in the core of the lipopolysaccharide of certain strains of *C. jejuni* may stimulate an immune response to this epitope in infected patients. It is hypothesized that this immune response may then lead to an autoimmune peripheral neuropathy because the Gm1 in the bacteria is identical to that in the nerve cell.

This study investigated the epidemiology of *Campylobacter* infection within the Tasmanian community by collecting isolates from patients notified to the Department of Health in Tasmania. This culture collection was then used to develop a robust method for speciation of isolates. This included development of a multiplex PCR for the hippurate gene/16S rRNA gene with subsequent dot blot hybridisation using labeled probes. Using this system, 237 enteritis strains were identified as *C. jejuni*, 10 *C. coli*, 2 *C. lari* and 1 *C. upsaliensis*.

C. jejuni serotype O:19 appears to be over-represented in most published studies of GBS but campylobacters of this serotype do not account for all *Campylobacter* isolates from GBS patients. Potential markers of GBS-associated campylobacters investigated in the present study included the use of a *Cholera* Toxin Binding Assay for Gm1-like epitopes in bacteria cell walls, a PCR for serotype O:19 strains and Pulsed Field Gel Electrophoresis (PFGE). While 33% of all Tasmanian strains were found to be Gm1 positive, no isolates were found to

belong to the serotype O:19. Further, computer-assisted analysis of PFGE profiles showed no similarity between GBS strains of *C. jejuni* and Tasmanian strains of *Campylobacter.*

Subtractive hybridisation was used to produce a DNA library containing sequences that are over-represented in GBS-associated strains of *C. jejuni*. This library has been used to screen GBS and non-GBS-associated strains of *C. jejuni*. A unique DNA sequence, present only in the GBS-associated strains, has been identified and a PCR-based assay developed to detect this gene (*Veh).* The gene has homology to a sequence (Cj1013c), determined as part of the recently completed *C. jejuni* genome sequencing project, which is a gene of unknown function. Further studies to define gene function have been hampered by the lack of a suitable mutagenesis system for *C. jejuni*. *Veh* has been cloned in a shuttle vector to enable its mobilisation into other transfer systems.

It is hoped that in the future, identification of *Campylobacter* strains containing the *Veh* gene will lead to prompt treatment of patients infected with these strains thereby decreasing the incidence of GBS and its associated morbidity and mortality.

CHAPTER ONE

LITERATURE REVIEW

1.1 Background

The genus *Campylobacter* was first proposed in 1963 (Sebald and Veron, 1963) to reclassify *Vibrio fetus* and "related vibrios"(King, 1957) that possessed distinct cultural characteristics separating them from the true vibrios. This original description contained just two species; *Vibrio fetus* and *Vibrio bubulus*. While the type strain for this new genus, *Campylobacter fetus*, had been known since the early 1900's as a cause of abortion and dysentery in cattle and domestic animals, it was not until 1947 that an association with human disease was made following their isolation from blood cultures (Vinzert, Dumas and Picard, 1947). It was discovered that the species *V. bubulus* differed from *V. fetus* in having both a high optimum growth temperature and a predominantly clinical presentation in those infected, of diarrhoea. Initial isolates of *V. bubulus* were obtained from blood cultures and it was proposed that isolation from faecal specimens would be possible if a culture medium could be made that suppressed the growth of normal flora of the intestinal tract (King, 1962). Over 10 years later in 1973, a study in Brussels used the fact that campylobacters are smaller in size than most other bacteria and by using a filter to capture the larger bacteria, campylobacters were cultured successfully for the first time from faecal samples in 5.1% of children with diarrhoea compared to only 1.3% of children without diarrhoea (Butzler *et al*, 1973). These results were confirmed, and a selective medium developed in 1977 which increased isolation rates of *Campylobacter* sp. to 7.1% in patients with diarrhoea (Skirrow, 1977). *Campylobacter jejuni* is now recognised as the most frequent bacterial agent of diarrhoea in developed countries, often exceeding the combined total for diarrheal illness caused by *Salmonella* sp., *Shigella* sp. and *Escherichia coli* 0157:H7 (Blaser, 1997).

With improved culture techniques, and soon after Skirrow's culture media was routinely used, a study was published describing the association of *Campylobacter* isolated from a patient with diarrhoea and the subsequent development of Guillian Barré Syndrome (GBS) (Rhodes and Tattersfield, 1982). Since that report numerous studies have shown the association between GBS and a preceding gastrointestinal infection with *Campylobacter jejuni* (Hughes and Rees, 1997).

Within 25 years of the first campylobacters being isolated from faecal samples, the genus had been expanded to include 21 species and subspecies. Smaller laboratories still perform culturing techniques as used in the late 1970's and 80's, and therefore only the common species are routinely isolated resulting in under-reporting of the true incidence of other

campylobacters causing diarrhoea and their possible link with neuropathies such as GBS. Recently a report was published describing the association of *Campylobacter upsaliensis* with the related neurological condition, acute motor axonal neuropathy (Ho *et al*, 1997) and the association of *Campylobacter jejuni* with other non-GBS neuropathies such as Miller-Fisher Syndrome (Salloway *et al*, 1996).

1.2 Reservoir of Infection

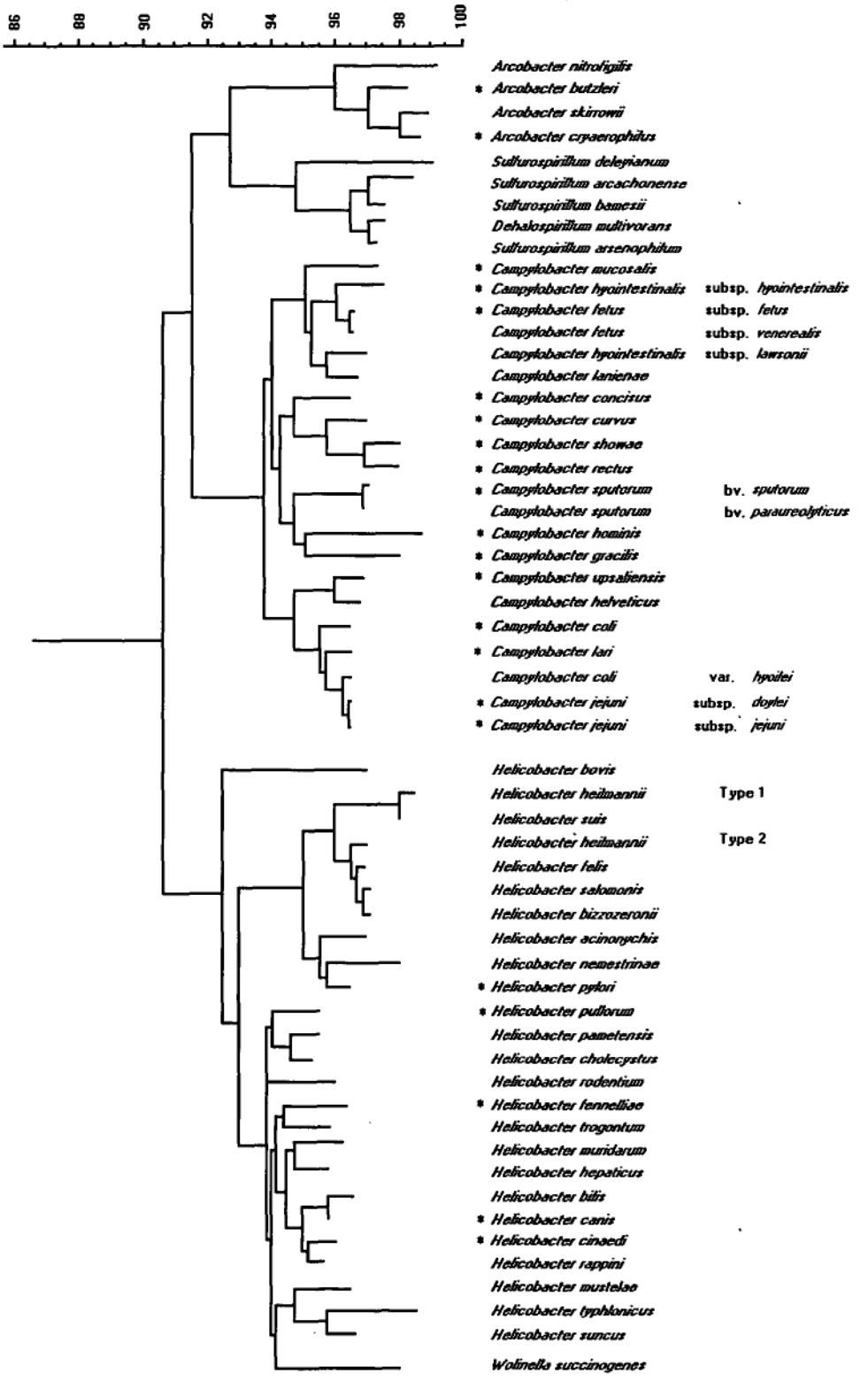
Campylobacter species and *Arcobacter* species are found in the intestinal tract of many domesticated and wild animals. Those animals used for food production such as poultry, cattle, sheep, swine and goats are therefore a major source of human infection. For example, *Campylobacter* species have been isolated in up to 80% of raw chickens sold in the UK (Corry and Atabay, 2001). Consumption of undercooked poultry or improper handling of raw meat are both possible sources of infection. Human to human transmission by a food handler with a diarrheal illness has also been documented (Olsen *et al*, 2001). The organisms can also be carried by human pets such as dogs, cats and birds (Blaser, 1997). Consumption of unpasteurised milk (MMWR, 2002) and contaminated water supplies (de Boer, 1996) have also been implicated.

1.3 Taxonomy

The taxonomy of the campylobacters and related bacteria has been extensively revised, particularly over the last decade. An extensive polyphasic study using DNA-rRNA hybridisations to determine relative phylogenetic positions with subsequent cross referencing of information with phenotypic and genetic data has produced the taxonomic structure currently in use (Vandamme *et al*, 1992). This study has grouped campylobacters into the rRNA superfamily VI comprising three main rRNA homology groups. Group one contains the genera *Campylobacter* (21 species and subspecies), group two, *Arcobacter* (4 species) while rRNA group three contains the genus *Helicobacter* (24 species) and the genus *Wolinella* (1 species) (On, 2001).

A separate group of saprophytic, sulpur-reducing strains of organisms has subsequently been added to the rRNA superfamily VI with 16S rRNA gene sequencing analysis clearly showing their close relationship to *Arcobacter* species (Figure 1.1). These organisms now belong to the new genus *Sulfurospirillum* which currently contains four species. Another closely related saprophytic bacterium isolated from activated sludge, *Dehalospirillum multivorans*, possesses many of the characteristics of the genus *Sulfurospirillum* and will likely be included in this new genus in the future (On, 2001).

Figure 1.1: Dendrogram showing relationships between members of the RNA Superfamily VI based upon 16S rRNA gene sequences and neighbor-joining clustering.



Sequences were obtained via Genbank and a multiple alignment obtained using BioNumerics software (Applied Maths, Kortrijk, Belgium) (Adapted from On, 2001)

* Species pathogenic for humans

1.3.1 Description of the pathogenic genera

Campylobacters are curved, S-shaped, or "seagull shaped" bacteria, 0.2 - 0.9 μm wide and 0.5 - 5.0 μm in length. Spherical or coccoid bodies may form in old cultures or in cultures exposed to air for prolonged periods (Nachamkin, 1995). They are non-spore forming Gram-negative bacilli and display a characteristic rapid darting motility. They may possess either a unipolar unsheathed flagellum or bipolar flagella (Blaser, 1989). The majority of the species require a microaerophilic atmosphere for growth at 37°C, while some species will only grow in anaerobic conditions at 37°C and others only in the presence of increased concentrations of hydrogen (On, 1996a). The G+C content of the DNA ranges from 29 to 45% (Versalovic and Fox, 1999).

Arcobacters are slightly curved, curved, S-shaped or "seagull shaped" bacteria that are 0.2 - 0.9 μm wide and 1.0 - 3.0 μm in length. As with campylobacters, arcobacters are motile but only ever display a unipolar unsheathed flagellum. This genus will grow at temperatures that are lower than required for *Campylobacter*, with most strains growing at 15°C, 25°C and 30°C, with variable growth at 37°C (Nachamkin, 1995). They also differ from the genus *Campylobacter* in being aerotolerant and in their fatty acid profiles (Lambert *et al*, 1987). All isolates described in a recent study of this genus grew in aerobic conditions at 25°C (On, 1996a).

Helicobacters are helical, curved or straight Gram-negative rods 0.3 to 1.0 μm wide and 1.5 to 5.0 μm long. They differ from campylobacters and arcobacters due to the presence of sheathed flagella. Some species possess a unipolar flagellum *(H. cinaedi and H. fennelliae)* or multiple unipolar or bipolar lateral flagella (*H. pylori*). The organisms are microaerophilic with optimum growth at 37°C. The G+C content of the DNA ranges from 30 to 48% (Versalovic and Fox, 1999).

1.3.2 Differentiation of species

As the organisms in RNA superfamily VI are asaccharolytic, very few tests are available for the differentiation of the individual species. Tests such as the optimum growth temperature and atmospheric growth conditions are therefore relied upon for speciation. Appendix A outlines the major differentiating characteristics and tests used for identification of organisms in this group that are pathogenic for humans.

1.4 Clinical Significance

The predominant infection by far seen in humans with this group of bacteria, is diarrhoea with abdominal pain, fever and sometimes vomiting. The organisms may occasionally be isolated from blood cultures, with rare extra-intestinal infections occurring in patients with some type of predisposing condition.

1.4.1 *Campylobacter jejuni* subspecies *jejuni*

C. jejuni subsp. *jejuni* is the most commonly isolated bacterial species associated with diarrhoea. Thus, it accounts for approximately 400 cases of reported gastroenteritis in Tasmania each year (an infection rate of approximately 1/1000) and approximately 11,000 throughout Australia. This rate is compared with 6,500 *Salmonella* and 700 *Shigella* cases for the same time period (CDI, 1997) and corresponds to infection rates seen in other developed nations such as the United Kingdom and USA (Nachamkin, 1995).

C. jejuni may be found comprising part of the normal intestinal flora of both wild and domesticated animals including most of the animals used in food production (eg. poultry, cattle, sheep and swine). The organisms can also be carried by human pets such as dogs, cats and birds (Blaser, 1997).

Although infections are seen throughout the year, there is a marked increase in prevalence in the summer months and early autumn. Infection occurs following ingestion of improperly cooked food (primarily poultry) but is also linked to contaminated milk and water (Tauxe, 1992). Infections display a bi-modal age distribution with the highest incidence seen in infants and young children with the second peak in adults 20 - 30 years old (Engberg *et al*, 2000). Epidemiological evidence shows that the majority of isolations are sporadic but that large outbreaks from a single strain have also been reported (Jones *et al*, 1993; Jackson *et al*, 1996).

The incubation period following ingestion of *C. jejuni*, calculated from outbreak investigations, is 24-72 hours, but incubation periods of greater than 1 week can occur (Blaser, 1997). Prodromal symptoms are often non-specific and can include headache, myalgias, chills and fever and may persist for more than 24 hours.

A spectrum of illness is seen associated with gastroenteritis, ranging from asymptomatic to severe. The major symptoms include abdominal cramping and fever followed by diarrhoea (with or without erythrocytes/leucocytes) lasting from several days to more than one week. The diarrhoea may initially be watery with more than eight bowel movements on the worst day of illness and will frequently contain blood. The abdominal pains associated with infection have been known to mimic acute appendicitis. Illness usually peaks over a 24-48

hour period, slowly improving and resolving over the following week but relapses have been reported in up to 20 % of cases (Blaser, 1997). Faecal excretion of *C. jejuni* can still occur during the convalescent stage. In a Norwegian study, carriage rates of *C. jejuni* averaged 37.6 days (range 15-69 days) (Kapperud *et al*, 1992). In another study, *C. jejuni* could be isolated from two thirds of patients at 2 weeks post-infection, from one third of patients at 4 weeks and from 4% at 6 weeks (Nachamkin, 1997).

Extra-intestinal infections also occur and may include bacteremia, reactive arthritis, bursitis, urinary tract infection, meningitis, endocarditis, peritonitis, erythema nodosum, pancreatitis, abortion and neonatal sepsis (Blaser, 1990). Guillain-Barré syndrome (GBS) appears to be strongly associated with *Campylobacter* infection as one of the sequelae of infection (Hughes and Rees, 1997).

Bacteremia occurs in 1.5/1,000 intestinal infections with the highest rate in the elderly (Skirrow *et al*, 1993) but with deaths being reported rarely (Tauxe, 1992).

Studies comparing developed and developing countries have shown a marked difference in features of *C. jejuni* infections. While in developed countries it is typical to have only 0-1 infections/lifetime seen principally in young adults, greater than 5 infections/lifetime are seen in developing countries with children less than 2 years of age being mainly affected. Widespread immunity is absent amongst adults in developed countries but present in adults in developing countries (Calva *et al*, 1988). Also in developed countries, illness manifests itself as inflammatory diarrhoea with the principal vehicle of transmission being under-cooked poultry. In developing countries, the illness manifests as a simple diarrhoea and the vehicle of transmission is unknown (Blaser, 1997).

As the majority of infections are self-limiting, antimicrobial intervention is not usually warranted. Treatment may be considered however where there is evidence of extra-intestinal infection, in compromised patients or persons with severe symptoms including bloody diarrhoea and/or fever. The current drug of choice in erythromycin but fluoroquinolones and tetracyclines are used as alternatives (Blaser, 1995).

1.4.2 *Campylobacter coli*

It is estimated that approximately 5-10% of cases of diarrhoeal illness caused by campylobacters are due to *C. coli*. (Nachamkin, 1995). Clinically, the illness caused by this species is indistinguishable from that seen with *C. jejuni*. This species has also been associated with indwelling device-related bacteremia (Hsueh *et al*, 1997). The species has been isolated from the intestinal tract of pigs, poultry, bulls, sheep and birds (On, 1996a).

1.4.3 *Campylobacter fetus* subspecies *fetus* (previously *Campylobacter fetus* subspecies *intestinalis*)

The first reported cases of human illness due to this species were in 1947 (Vinzert, Dumas and Picard, 1947). A distinguishing feature of this subspecies, and the most common presentation in humans, is bacteremia. In a review of 91 cases of bacteremia, 50 were due to *C. fetus*, 10 due to *C. jejuni* and the remaining 31 of uncertain speciation. The predominant clinical presenting features in patients with *C. fetus* bacteremia were fever in 96% of patients, diarrhoea/nausea/vomiting/abdominal pain in 38%, hepatosplenemegaly/jaundice in 44%, a mortality rate of 26%, with 96% of patients > 14 years of age and having an underlying disease process in 72% of the time (Guerrant *et al*, 1978). Compare this with bacteremia due to *C. jejuni* (diarrhoea/nausea/vomiting/abdominal pain being the predominant feature in 80% of cases, fever in 60%, hepatosplenamegaly/jaundice in 10%, mortality of 20%, only 30% > 14 years old with the majority: 60% being < 1 year old (Guerrant *et al*, 1978).
C. fetus also has a predilection for the vascular endothelium. There have been 15 reported cases of *C. fetus* associated aneurysms predominantly in the abdominal aorta (Montero *et al*, 1997), endocarditis (Loeb *et al*, 1966) and thromophlebitis (Carbone, Heinrich and Quinn, 1985).
Other extraintestinal infections include spontaneous peritonitis (Elcuaz *et al*, 1998), septic abortion (Rettig, 1979), abscesses, meningitis, septic arthritis and salpingitis (Mishu, Patton and Tauxe, 1992) and prosthetic hip joint infections (Yao, Ng and Campbell, 1993).

1.4.4 *Campylobacter upsaliensis* (previously CNW *Campylobacter*)

A thermophilic *Campylobacter* was first isolated from dog faeces in 1983. It was subsequently isolated from humans with gastroenteritis (Goossens *et al*, 1990). Isolation from a breast abscess (Gaudreau and Lamothe, 1992) and from a case of abortion have also been reported (Gurgan and Diker, 1994). While most patients that develop gastroenteritis from *C. jejuni* and *C. coli* acquire their infections from improperly cooked poultry, the reservoir of *C. upsaliensis* seems to be the family pet, with carriage rates of 5% in cats and 29% in dogs (Hald and Madsen, 1997). Possible further evidence for this comes from a reported case involving the isolation of *C. upsaliensis* from the faeces of a patient with diarrhoea and from his healthy dog. Both isolates were shown to be the same strain by plasmid analysis, protein profiles, immunotyping, antibiotic susceptibility pattern and phenotypic characterisation (Goossens, Vlaes and Butzler, 1991).

1.4.5 *Campylobacter lari*
(previously *Campylobacter laridis*, 'NARTC')

In 1980, a new species was proposed for those campylobacters that differed from other thermophilic strains by their resistance to nalidixic acid and halotolerance (Skirrow and Benjamin, 1980). The species name *C. laridis*, later changed to *C. lari*, was chosen as the majority of isolates were obtained from seagulls (genus *Larus*) (Benjamin *et al*, 1983). Although the organism had been isolated from a number of different avian and mammalian species, it had not been associated with illness (Roberts, 1983). In 1985, a report was published describing six cases of human illness associated with *C. lari*; four cases of enteritis, one with severe cramping abdominal pain and one of terminal bacteremia in an immunocompromised host (Tauxe *et al*, 1985). While extra-intestinal infections are rare, a fatal case of septic shock was recently reported in an 81 year old immunocompetent man with a prosthetic joint infection and bacteremia caused by this species (Werno *et al*, 2002). Large waterborne infections affecting over 100 individuals have also been described (Nachamkin, 1995).

1.4.6 Other pathogenic *Campylobacter* and *Arcobacter* species

While early isolations of *Campylobacter concisus* were found from the human oral cavity (Tanner *et al*, 1981) and increased numbers were associated with inflamed gingival crevices (Moore *et al*, 1985), the pathogenic role of this species in periodontal disease has remained unclear. Subsequently, published reports of *C. concisus* from faecal samples in patients with gastrointestinal disorders began to emerge (Vandamme *et al*, 1989) but years later the same research group questioned the role of *C. concisus* in gastrointestinal disorders when no statistically significant difference was found in isolation rates from children with diarrhoea compared with the control group (13.2% vs 9% respectively). A large number of those strains tested were isolated from children attending a day care centre. DNA fingerprinting using arbitrary primers showed that the majority of children harboured their own unique strain of *C. concisus* and the prevailing diarrhoea was not due to an epidemic strain (Van Etterijck *et al*, 1996). Confirmation of the status of *C. concisus* as a normal commensal of the human gut was later reported in a study in which 45 strains of *C. concisus* were isolated in nearly equivalent proportions from 1,376 faecal samples from both patients with diarrhoea and healthy controls (Engberg *et al*, 2000). This species has however been implicated as a cause of diarrhoea in immunocompromised AIDS patients (Snijders *et al*, 1997).
Another species implicated in gastroentestinal infections is *Campylobacter hyointestinalis* subspecies *hyointestinalis*, isolated from humans experiencing watery, non-bloody diarrhoea

and ranging in age from 8 months to 79 years old (Edmonds *et al*, 1987) as well as from cases of proctitis in homosexual males (Fennell *et al*, 1986).

Campylobacter mucosalis (previously *Campylobacter sputorum* subspecies *mucosalis*), a well documented cause of porcine necrotic enteritis and ileitis (On, 1996a), has also been isolated from two children (12 and 18 months of age) presenting with mild diarrhoea and loose stools but without gross blood in faeces (Figura *et al*, 1993) while *Campylobacter jejuni* subspecies *doylei* (previously 'NNC group'), first isolated in Australia in 1985 from children less than 5 years of age with diarrhoea (Steele, Sangster and Lanser, 1985), has also been reported from AIDS patients with diarrhoea (Snijders *et al*, 1997).

Consumption of contaminated water during travel and person-to-person transmission have resulted in human enteritis being attributed to *Arcobacter butzleri* (previously *Campylobacter butzleri*) (Kiehlbauch *et al*, 1991a). Cases of bacteremia, endocarditis and peritonitis have also been reported (Nachamkin, 1995). This species has been reported to comprise up to 16% of all campylobacter-like organisms causing diarrhoea (Taylor *et al,* 1991) but is frequently not isolated as optimum growth conditions have yet to be determined (Nachamkin, 1995).

The second pathogenic species of this genus, *Arcobacter cryaerophilus* (previously *Campylobacter cryaerophilia*), has also been reported to cause diarrhoea and bacteremia in humans (Nachamkin, 1995). It has been isolated from the gastrointestinal tract of pigs, bulls, sheep, horses and found in sewage (On, 1996a).

A number of *Campylobacter* species have been associated with periodontitis. *C. rectus* has been isolated from the complex subgingival flora found in cases of periodontitis along with *Campylobacter gracilis* (previously *Bacteroides gracilis*) (Tanner *et al*, 1981) as has *Camplyobacter showae* (previously *Wolinella curva* subspecies *intermedius*) and *Campylobacter curvus* (previously *Wolinella curva*) (On, 1996a).

Extra-intestinal infections of these uncommonly isolated species of *Campylobacter* are rare. *C. curvus* was isolated from lung, jaw and neck infections (Finegold and Jousimies-Somer, 1997) as well as from peritoneal fluid (Wexler *et al*, 1996). *C. rectus* (previously *Wolinella recta*) has been isolated in association with *Actinomyces viscosus* from a patient with an actinomycotic chest wall mass (Spiegel and Rtelford, 1984).

A number of isolations of *C. gracilis* have been reported from serious deep-seated infections such as soft tissue abscesses, appendicitis/peritonitis/intra-abdominal abscesses, parapharyngeal abscess, and empyema as well as bacteremia and wound infections (Molitoris, Wexler and Finegold, 1997).

Campylobacter sputorum biovar *sputorum* is regarded as a commensal of the human gastrointestinal tract, with clinical isolations being rare. Published reports include isolation from a leg abscess, another from a case of infantile diarrhoea (Roop *et al*, 1985) and from an

axillary abscess (On *et al*, 1992). Isolation from AIDS patients with diarrhoea has also been noted (Snijders *et al*, 1997).

1.4.7 Non pathogenic campylobacters and arcobacters

The following species have not been associated with any known human diseases to date:

Campylobacter fetus subsp. *veneralis*
Campylobacter coli var. *hyoilei*
Campylobacter hyointestinalis subsp. *lawsonii*
Campylobacter sputorum biovar *fecalis*
Campylobacter sputorum biovar *paraureolyticus*
Campylobacter helveticus
Campylobacter lanienae
Arcobacter nitrofigilis
Arcobacter skirrowii

1.5 Techniques for isolation

1.5.1 Selective agars

Many different formulations have been described since the first selective agar was produced by Skirrow in 1977. The earlier media containing blood were subsequently found to be inhibitory to the growth of campylobacters and so were replaced by charcoal based media containing a combination of antimicrobial agents to which *C. jejuni* and *C. coli* are resistant. These include as the selective agent, antifungals such as amphotericin B, anti-Gram positive bacterial agents such as vancomycin and anti-coliform agents such as cephalothin or cefoperazone. Over the following years, other non *C. jejuni/coli* campylobacters were found to be associated with gastroenteritis. Isolation of many of these new species was impeded by the fact that they were susceptible to the new selective agarose formulae. The true incidence and clinical spectrum of the newer campylobacters is therefore yet to be established as most laboratories routinely culture for *C. jejuni* and *C. coli* only on commercially available media. The antibiotics in the more widely used media formulations are given in Table 1.1 and the ability of various routine selective media in isolating members of the genus *Campylobacter* and *Arcobacter* are summarised in Table 1.2.

A number of different studies have examined the efficacy of these selective media for the isolation of *C. jejuni* and *C. coli*. One study screened 1,980 faecal specimens from patients with gastroenteritis and found 161 *Campylobacter* strains (148 *C. jejuni*, 12 *C. coli*, 1 *C. upsaliensis*) (Endtz *et al*, 1991a). The three media used in this study: Skirrow medium (SKM), Charcoal cefoperazone deoxycholate agar (CCDA) and Charcoal based selective agar (CSM) enabled the highest isolation rates of campylobacters, with no statistical difference between these three formulations (80%, 83% and 87% of strains isolated respectively). Each

of the media examined did not necessarily grow the same strains and the best isolation rates were produced with a combination of media and filtration methods. CSM and CCDA were also found to have the highest rates of isolation of *C. coli*. The lowest recovery rate of campylobacters was found when using the filtration method with only 61% of campylobacters being detected. This method however was the only one to grow the single *C. upsaliensis* detected. This study also found that the two media, CSM and CCDA, possessed the lowest number of faecal contaminants allowing for easier recognition of *Campylobacter* organisms.

Several of the selective media such as blood free, semi-solid motility medium (SSM) and SKM were devised for use at 42°C and have poor selective qualities at 37°C, whereas CSM and CCDA show good selective properties at 37°C (Endtz *et al,* 1991a).

1.5.2 Enrichment broths

The use of an enrichment broth is extremely useful when trying to isolate low numbers of campylobacters from convalescent GBS patients. In a study by Taylor et al (1988), dramatically improved isolation rates were obtained in GBS patients showing an increase of 31% over conventional plating techniques when testing patients more than 20 days after the onset of gastroenteritis.

A number of enrichment broth formulations have been produced for the recovery of campylobacters. These include: Campy-thio broth [CT] (Blaser *et al*, 1979), *Campylobacter* enrichment broth [CEB] (Martin *et al*, 1983), Preston enrichment broth (Bolton and Robertson, 1982), Bruce-Zochowsky medium [BZ] (Hodge and Terro, 1984), Alkaline peptone water [APW] (Phillips and Nash, 1985) and Semi-solid motility media [SMTM] (Chan and MacKenzie, 1982).

A number of comparative studies have been published. Thus Martin *et al* (1983), improved isolation rates by 46.3% on samples kept at 4°C for 3 weeks with CBE enrichment broth over the commonly used enrichment broth at that time, Campy-thio broth [CT]. Agulla *et al* (1987), compared CT, APW, CEB and BZ. Unlike a previous study by Martin *et al* (1983), their four enrichment broths did not improve isolation rates from fresh faecal samples of acute gastroenteritis patients. They proposed that enrichment broths would only be useful when attempting to detect low numbers of viable organisms. They did however note that CEB and BZ produced slightly better isolation rates compared to the other media tested.

Table 1.1: Antibiotic supplements used in selective agars for *Campylobacter* species

MEDIA	ANTIBIOTIC SUPPLEMENT	REFERENCE
Skirrow medium [SKM]	Vancomycin (10 mg/L), Trimethoprim (5 mg/L), Polymyxin (2,500 IU/L)	Skirrow, 1977
Charcoal cefoperazone deoxycholate agar [CCDA]	Cefoperazone (32 mg/L)	Bolton, Hutchinson and Coates, 1984
Charcoal based selective agar [CSM]	Vancomycin (20 mg/L), Cefoperazone (32 mg/l), Cycloheximide (100 mg/L)	Karmali *et al*, 1986
Blood free, semi-solid motility medium [SSM]	Cefoperazone (32 mg/L) Trimethoprim lactate (50 mg/L)	Goossens *et al*, 1989
Campy BAP [BAP]	Vancomycin (10 mg/L), Trimethoprim (5 mg/L), Polymyxin (2,500 IU/L), Cephalothin (15 mg/L), Amphotericin B (2 mg/L)	Blaser *et al*, 1979
Cefoperazone,amphotericin Teicoplanin [CAT]	Cefoperazone (8 mg/L), Amphotericin B (10 mg/L), Teicoplanin (4 mg/L)	Engberg *et al*, 2000

Table 1.2: Growth of *Campylobacter* species and *Arcobacter* species on various selective agar

ORGANISM	SKM	CCDA	CSM	SSM	BAP	FILTER
C. jejuni subsp. *jejuni*	G	G	G	G	G	G
C. jejuni subsp. *doylei*	*	X	X	X	X	G
C. coli	G	G	G	G	G	G
C. fetus subsp. *fetus*	*	G	G	G	X	G
C. fetus subsp. *venerealis*	*	*	*	*	X	G
C. lari	*	G	G	G	G	G
C. upsaliensis	*	X	X	X	X	G
C. hyointestinalis subsp. *hyointestinalis*	*	X	X	X	X	G
C. hyointestinalis subsp. *lawsonii*	*	*	*	*	*	G
C. concisus	*	X	X	X	X	G
C. curvus	*	*	*	*	*	G
C. rectus	*	X	X	X	X	G
C. gracilis	*	X	X	X	X	G
C. mucosalis	*	X	X	X	X	G
C. sputorum bv. *sputorum*	*	*	*	*	X	G
C. sputorum bv. *bubulus*	*	*	*	*	X	G
C. sputorum bv. *fecalis*	*	*	*	*	X	G
C. showae	*	*	*	*	*	G
C. helveticus	*	*	*	*	*	G
C. hyoilei	*	*	*	*	*	G
A. nitrofigilis	*	*	*	*	X	G
A. butzleri	*	G	G	G	G	G
A. skirrowii	*	*	*	*	*	G
A. cryaerophilus	*	G	G	G	G	G

X: NO GROWTH, G: GROWTH, *: UNKNOWN
Adapted from (Endtz *et al*, 1991a) (On, 1996a)

Sjogren, Lindblom and Kaijser (1987) evaluated SMTM against CEB as both an enrichment broth and transport medium. They found that SMTM improved isolation by 8% over the other media. Another advantage noted was the ability of campylobacters to survive for long periods in SMTM thus emphasising the usefulness of this medium as a transport medium. After initial inoculation and incubation at 42°C for 24 hours, viable organisms were still found after 1 month at room temperature. Also the enrichment culture yielded a more luxuriant growth as compared to isolation on primary plating (Chan and MacKenzie, 1982).

1.5.3 Filtration

Butzler *et al* (1973) first used membrane filters to isolate campylobacters from faecal samples. They noted that due to the small size of these bacteria, they could pass through 0.45 μm and 0.65 μm cellulose filters while the larger enteric bacteria were trapped. The method involves placing a filter on the surface of a non-selective agarose plate such as horse blood agar, making a suspension in *Brucella* broth and placing 10 drops of the solution onto the filter surface. After the fluid has passed through the filter, it is then discarded and the agar plate incubated normally. A large study by Steele and McDermott (1984) of 1000 diarrhoea faecal samples, found that the filtration system isolated all 45 of the campylobacters grown on their selective agarose plates at 42°C with five addition non-C. *jejuni Campylobacter* strains being isolated by the filter system alone.

In another study, 0.65 μm cellulose acetate membrane filters were found to be superior to 0.45 μm cellulose triacetate filters for the recovery of campylobacters (Wells *et al*, 1989). Early studies isolating *C. upsaliensis* from blood cultures of bacteremic paediatric patients with gastroenteritis, failed to isolate campylobacters using routine media directly from faecal samples as the strains were susceptible to the antibiotics employed in routine agarose plates at that time (Lastovica, Le Roux and Penner, 1989). Some investigators have used modified agarose formulae to grow *C. upsaliensis* (Walmsley and Karmali, 1989) but poor growth of *C. upsaliensis* on the newer media is still being reported (Hald and Madsen, 1997). Current studies such as the incidence of campylobacters in AIDS patients with enteritis, still rely on the filtration technique for the isolation of *C. upsaliensis, C. concisus* and *C. mucosalis* (Snijders *et al*, 1997).

One of the major disadvantages of the filtration system is that a larger number of organisms are required for growth as compared to selective agars. It has been found that $> 10^5$ cfu/ml of campylobacter bacteria are required for detection; much more than the required number for selective agars (Goossens *et al*, 1990).

1.5.4 Growth Conditions

1.5.4 (a) Incubation temperature

The family Campylobacteraceae possesses many members with varying optimal growth temperatures. The arcobacters tend to grow better at lower temperatures such as 25-30°C, thermophilic campylobacters proliferate at 42°C with the remainder of 12 or so other species and subspecies growing best at 37°C.

As it has been believed for many years that only the thermophilic group of campylobacters (*C. jejuni* subsp. *jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) cause human disease, most laboratories only incubate selective media at 42°C. Species that have been associated with diarrhoea that would not grow at this temperature include *C. jejuni* subsp. *doylei*, *C. fetus* subsp. *fetus*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. concisus*, *C. sputorum* biovar *sputorum*, *A. butzleri* and *A. cryaerophilus*.

Table 1.3 summaries the incubation temperature range and atmospheric gas requirements for the growth of campylobacters and arcobacters from a number of different studies.

1.5.4 (b) Incubation time

While it is still common practice in many diagnostic laboratories to incubate selective media for only 48 hours, some studies have found that incubating for up to 72 hours increases yields. In one such study, the rate of isolation was increased by an average of 18% across all the selective media tested (Endtz *et al*, 1991a). Growth of the slower growing campylobacters, such as *C. mucosalis*, requires incubation for up to 5 days.

1.5.4 (c) Atmospheric gas conditions

The majority of campylobacters will grow in a microaerophilic atmosphere containing ~ 5 % O_2, 10 % CO_2 and 85 % N_2. Some *Campylobacter* species such as *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curva*, *C. rectus* and *C. hyointestinalis* may also require hydrogen in the gas mixture for isolation. As some studies have shown variable concentrations of hydrogen in gas mixtures when using commercial gas-packs, an evacuation and replacement method with a bottle gas of known concentration is recommended (Kiehlbauch *et al*, 1995).

Table 1.3: Incubation temperature and atmospheric gas mixture for growth of *Campylobacter* and *Arcobacter* species

ORGANISM	mO_2 15°C	mO_2 25°C	O_2 25°C	mO_2 30°C	O_2 37°C	mO_2 37°C	ANO_2 37°C	mO_2 42°C
C. jejuni subsp. *jejuni*	-	-	01	*	01	99	01	+
C. jejuni subsp. *doylei*	-	-	01	*	01	99	01	-
C. coli	-	-	01	*	01	99	01	+
C. fetus subsp. *fetus*	-	+	01	*	01	99	25	-
C. fetus subsp. *venerealis*	-	+	01	*	01	99	73	-
C. lari	-	-	01	*	01	99	01	+
C. upsaliensis	-	-	01	*	01	99	01	+
C. hyointestinalis subsp. *hyointestinalis*	-	+	01	*	01	99	01	*
C. hyointestinalis subsp. *lawsonii*	-	+	*	*	*	*	*	*
C. concisus	-	-	01	(+)	01	99	99	(-)
C. curvus	-	-	*	*	*	*	*	*
C. rectus	-	-	01	*	01	01	99	*
C. gracilis			01	*	01	01	99	*
C. mucosalis	-	-	*	+	-	+	+	(+)
C. sputorum bv. *sputorum*	-	-	*	+	-	+	+	+
C. sputorum bv. *bubulus*	-	-	*	+	-	+	V	+
C. sputorum bv. *fecalis*	-	-	*	V	-	+	+	+
C. showae	-	-	*	*	*	*	*	*
C. helveticus	-	-	*	*	*	*	*	*
C. hyoilei	*	*	*	*	*	*	*	*
A. nitrofigilis	+	+	*	*	*	*	*	-
A. butzleri	+	+	99	*	99	99	99	V
A. skirrowii	+	+	*	*	*	*	*	V
A. cryaerophilus	+	+	99	*	50	58	16	-

- 01-99 : percentage positive, +: positive result, -: negative result
- (+) 70-90% positive, (-) 20-33% negative, V: 50-66% positive.
- mO_2: microaerophilic, O_2: aerobic, ANO_2: anaerobic
- *: not known

Adapted from (On *et al*, 1992), (On, 1996a), (Nachamkin, 1995)

1.5.5 Detection by Polymerase Chain Reaction (PCR)

While the application of PCR to the detection of organisms in faeces is problematic due to the presence of PCR inhibitors, the advantages of decreased turn around time and the detection of bacteria in a metabolic state less amenable to culture on selective media (ie sub-lethally damaged cells, viable but non-culturable cells or even dead cells) have encouraged researchers to apply PCR to the direct detection of *Campylobacter* species in faeces. In one such study, primers were designed to detect and differentiate *C. jejuni* and *C. coli*. All 20 culture positive isolates (18 *C. jejuni* and 2 *C. coli*) were detected and correctly identified (Linton *et al*, 1997).

A large scale PCR-based study utilising a detection and identification algorithm consisting of a screening PCR and subsequent species identification by PCR-enzyme-linked immunosorbent assay was applied to 3,378 faecal samples (Lawson *et al*, 1999). Results from this study showed that the sensitivity of PCR based methods was comparable to current culture techniques with PCR yielding 492 positive samples compared with 464 positive cultures. In addition, mixed infections with *C. jejuni* and *C. coli* were detected in 19 samples and 1 with *C. jejuni* and *C. upsaliensis* that was not apparent from culture. Detection rates of the infrequently isolated strains of *Campylobacter* species were also increased using PCR. Of the 11 samples PCR positive for *C. upsaliensis* , only 2 were detected by culture. Three samples were also PCR positive for *C. hyointestinalis* but only 1 culture positive.

1.6 Epidemiological investigations

1.6.1 Serotyping

Two major serotyping schemes are used for the campylobacters. The first, described by Lior *et al* (1982), detects heat-labile bacterial surface antigens such as flagella and can detect >100 serotypes of *C. jejuni*, *C. coli* and *C. lari*. The second is the O typing system of Penner *et al*, (1980) which is based upon the heat-stable lipopolysaccharide antigens. This system can detect 60 types of *C. jejuni* and *C. coli* (Patton and Wachsmuth, 1992).
Using these techniques it has been shown that *C. jejuni* strains associated with GBS are confined to a relatively small number of serotypes, namely O:1, O:2, O:10, O:19, O:41 and O:64. In one Japanese study by Kuroki *et al* (1993), it was found that 10 of the 12 strains tested belonged to serotype O:19. In another study from USA, serotype O:19 accounted for 35% of strains tested from GBS patients, significantly higher than the 2% normally found in patients with uncomplicated gastroenteritis (Mishu, Patton and Blaser, 1993).
Serotyping is performed in only a few reference laboratories because of the time and expense needed to maintain quality serotyping anti-sera. While commercially available heat-labile antigens are available, they have been shown to be of poor quality (Nicholson and Patton, 1993).

1.6.2 Ribotyping

Polymorphism of DNA coding for 16S rRNA (ribotyping) has been used successfully to discriminate between stains of *C. jejuni* subsp. *jejuni, C. jejuni* subsp. *doylei, C. coli, C. lari, C. fetus* subsp. *fetus* and *C. upsaliensis* (Moureau *et al*, 1989) as well as separating arcobacters from campylobacters (Kiehlbauch *et al*, 1991b). Its discriminating power has

been shown to surpass serotyping, the "gold standard" of epidemiological typing schemes for the genus *Campylobacter* (Fitzgerald, Owen and Stanley, 1996) but not the newer DNA techniques of Pulsed Field Gel Electrophoresis (PFGE) and PCR.

1.6.3 Pulsed Field Gel Electrophoresis (PFGE)

Genomic DNA digested with infrequent cutting restriction endonucleases and resulting fragment separation by PFGE has proved to be a valuable epidemiological tool. The technique has been used for typing *C. jejuni* in outbreaks of gastroenteritis and to detect the source of infection (Hanninen *et al*, 1998; Gibson, Lorenz and Owen, 1997; Olsen *et al*, 2001) and for typing *C. fetus* (Rennie *et al*, 1994; Fujita *et al*, 1995) and *C. hyointestinalis* (Salama *et al*, 1992). The technique has been shown to be more discriminative than the Preston Biotyping scheme utilised in many reference laboratory as an epidemiological tool when typing *Campylobacter* strains (Dawkins *et al*, 1994; Jones *et al*, 1993) and when compared to serotyping (Gibson, Lorenz and Owen, 1997). A recent study in Denmark compared PFGE; Penner heat-stable serotyping; automated ribotyping (RiboPrinting); random amplified polymorphic DNA typing (RAPD); restriction fragment length polymorphisms of the flagellin gene, *fla* A (*fla*-RFLP) and denaturing gradient gel electrophoresis of *fla* A (*fla*-DGGE). A collection of 90 isolates of *C. jejuni* from poultry, cattle, and sporadic human clinical cases as well as strains from a waterborne outbreak of gastroenteritis were tested. While all methods identified the outbreak strain, when comparing unrelated strains, RAPD and PFGE were found to be the most discriminatory methods followed by *fla*-RFLP, RiboPrinting, *fla*-DGGE and serotyping (Nielsen *et al,* 2000). Genetic methods have begun to be employed to analyse non-related GBS strains of *C. jejuni* from around the world. While some studies have shown the clonal nature of some serotypes associated with GBS (Wassenaar *et al*, 2000), studies utilising various genetic methods to determine a possible serotype-independent epidemiological marker have been rare. A broad scale study involving 11 GBS strains from China, Denmark, Mexico, Thailand, United Arab Emirates and USA were compared to 47 control strains from cases of gastroenteritis. The molecular analysis was performed with Multilocus Enzyme Electrophoresis (MLEE), PFGE, RiboPrinting and *fla* typing (Endberg *et al*, 2001). This study which contained GBS-associated strains from serotype HS: 2, HS: 4, HS: 5, HS: 19, HS: 37, HS: 41 and HS: 53 with controls from the same serotypes, showed that GBS-associated strains are a heterogenic population of strains and do not constitute a unique population across serotypes. For example, dendrograms of PFGE results clearly showed GBS-related strains being spread amongst control strains.

1.6.4 PCR based

A number of different PCR based epidemiological typing schemes have been developed. Fujimoto *et al* (1997), used PCR to amplify the flagellin gene (*flaA*) with subsequent digestion with restriction endonucleases *Dde*I, *Mbo*I, *Mse*I and *Alu*I to detect differences in *C. jejuni* strains isolated from patients with Guillain-Barré Syndrome and patients suffering from gastroenteritis. Genetic variability using the flagellin genes (*flaA* and *flaB*) was confirmed in an Egyptian study which found that serogroups could be further subdivided using this method (Mohran *et al,* 1996). A report by Harrington, Thomson-Carter and Carter (1997) demonstrated recombination within and between flagellin loci of natural strains suggesting that a typing scheme based on restriction fragment length polymorphism analysis of PCR-amplified flagellin genes cannot be considered as a stable method for long-term monitoring of pathogenic *Campylobacter* strains.

Differentiation of strains by random amplified polymorphic DNA (RAPD) analysis has proved to be a useful PCR technique in a number of *Campylobacter* studies (Madden, Moran and Scates, 1996; Hilton *et al*, 1997).

Comparison of serotype O:19 *C. jejuni* strains associated with GBS and from patients with gastroenteritis have been typed using RAPD PCR analysis and shown to be closely related to one another. In contrast, other serotypes show great variability with this technique (Fujimoto *et al*, 1997). This study identified a 1.4 kb fragment which was specific for serotype O:19. This was later developed into a PCR technique to specifically identify O:19 strains from other serotypes allowing for rapid identification of a serostrain seen more regularly in GBS patients than other serotypes (Misawa, Allos and Blaser, 1998).

1.6.5 Multi Locus Sequence Typing (MLST)

With decreasing costs of DNA sequencing, epidemiological methods based upon sequence data would inevitably follow. One such method which compares genetic variation in sequence data from seven house-keeping genes, is Multi Locus Sequence Typing (MLST). Typically, nucleotide sequences of ~450 bp internal fragments of these seven loci are determined and the sequence type (ST) defined by the alleles present at the seven loci (Day *et al*, 2001). Population lineages can then be determined by the extent of the variation within these alleles (Pitt and Saunders, 2000). This technique can be performed on live or killed bacteria and purified DNA extracts. Being an easily standardised technique, MLST information can be easy stored and compared between laboratories with dedicated web sites already storing MLST data and protocols (http://mlst.zoo.ox.ac.uk) (Dingle *et al,* 2001).

A recent study utilised MLST to characterise isolates of *Staphylococcus aureus* from asymptomatic nasal carriage and from episodes of severe disease (Day *et al*, 2001). Their results showed that isolates causing disease were not drawn randomly from the carriage population, but were usually confined to a number of distinct MLST groups.
Another study evaluated the newer molecular subtyping approaches (Multilocus DNA Fragment Typing [MLDF], MLST, sequencing of 16S rRNA gene and PFGE) in a *Neisseria meningitidis* outbreak setting. With 16S rRNA gene sequencing and PFGE providing the highest sensitivity and specificity of the typing methods studied, both tests were adopted into their epidemiological protocol (Mayer *et al*, 2002).

1.7 Guillain-Barré Syndrome

1.7.1 Clinical Features

Guillain-Barré Syndrome (GBS) covers a group of clinical syndromes in which idiopathic peripheral neuropathy causes acute or sub-acute weakness of at least two limbs. This progresses and plateaus within 4 weeks of presentation. Previous studies had found that > 95% of cases present in this manner and patients with neuropathies progressing past 4 weeks are delineated chronic demyelinating polyradiculoneuropathies (Ashby *et al*, 1978).
GBS normally affects the motor, sensory and autonomic nerves supplying the limbs and may include the respiratory muscles and facial, bulbar and ocular motor nerves. Symptoms typically begin with weakness, sensory disturbance or pain and most patients develop rapidly progressive symmetrical weakness, that is worse in lower limbs than the upper limbs. Most patients experience numbness, tingling and pain and many have bladder disturbances, facial weakness and difficulty swallowing. CSF protein levels are raised in 80% of patients. Autonomic disturbances are also common with fluctuations in heart rate, blood pressure and bowel function (Hadden and Gregson, 2001).
While GBS is a self-limiting disease in the majority of patients, the condition does have a mortality rate of ~ 10% and ~ 25% of patients require artificial ventilation. While plasma exchange and intravenous therapy have hastened early recovery, up to 10% of patients are still left with severe disabilities up to 48 weeks later (Hughes and Rees, 1997).
Until recently, GBS was defined as a single homogenous clinical entity but researchers now believe that the syndrome can be separated into four distinct groups:

- Acute inflammatory demyelinating polyneuropathy (AIDP); the most frequently encountered pattern of GBS seen in Europe, North America and Australia (Asbury, 2000), is characterised by an immune-mediated attack on myelin with varying degrees of

lymphocytic infiltration. With severe cases of AIDP, there is also axonal degeneration (Griffin *et al*, 1996).

- Acute motor-sensory axonal neuropathy (AMSAN); previously called axonal GBS in which the axon appears to be the target of the immune system, is found less frequently than AIDP in Europe and North America but is common in China (Griffin *et al,* 1995) and Japan (Yuki *et al*, 1990). This form is usually severe involving both the motor and sensory fibres.

- Acute motor axonal neuropathy (AMAN); a more benign form of GBS which presents with mainly motor neuron involvement (Griffin *et al*, 1996).

- Miller-Fisher syndrome (MFS), is characterised by acute onset of unsteadiness of gait (ataxia), loss of reflexes (areflexia) and an inability to move the eyes, usually associated with non-reactive pupils (opthalmoplegia) (Fisher, 1956).

1.7.2 Epidemiology of GBS

A number of epidemiological studies from around the world have been published over the last 40 years. Annual prevalence rates range throughout these studies from 0.4 to 4 cases per 100,000 population (median, 1.3) (Hughes and Rees, 1997). A study in Australia by Storey *et al* (1989), had an prevalence rate of 0.9/100,000.

In the vast majority of these studies, males are more commonly affected than females (~ 1.25:1) with a bimodal age distribution having a peak in young adults and also in the elderly. The incidence has been found to be lower in children than adults, with the highest in the elderly, possibly due to failure of suppression autoimmunity in this age group.

Other studies have found that the AIDP form of GBS appears to affect an older population in Western countries, whereas the AMAN form tends to be found primarily in children and young adults (McKhann *et al*, 1993).

While most studies have not been able to identify a seasonal relationship for GBS, studies in northern China have shown annual summertime peaks with the AMAN form of GBS (Ho *et al*, 1995).

Up to two thirds of cases of GBS have an antecedent illness. Of these, respiratory infections are found to be the most commonly reported antecedent illness followed by gastrointestinal infections, of which *C. jejuni* is the most frequently identified cause (Hughes and Rees, 1997). A documented outbreak of *Campylobacter jejuni* gastroenteritis in Jordan during

1978 affecting >5,000 people subsequently produced 16 cases of GBS, 8 to 24 days after the onset of diarrhoea (Khoury, 1978). In contrast to the age distribution of GBS, *Campylobacter* spp. infections in developed countries are found at slightly higher rates in males compared to females and a bimodal age distribution with peaks in the <1 year old and a second lower but broader peak in the 15 to 30 years of age (Blaser, 1997).

Infections with Cytomegalovirus, Epstein-Barr virus, Varicella-zoster virus and *Mycoplasma pneumoniae* have also been implicated (Hadden and Gregson, 2001) . GBS following vaccination has also been reported with rabies and swine influenza vaccines (Hughes and Rees, 1997).

1.7.3 *Campylobacter jejuni* and GBS

The first reported case of GBS associated with *Campylobacter* infection was published by Rhodes and Tattersfield (1982).

1.7.3 (a) Serological studies

As reports suggest that the median duration of excretion of *Campylobacter* in stools of infected patients is only 16 days and GBS patients present 1 to 3 weeks post infection, the majority of studies have relied upon serological assays to determine an association between *Campylobacter* and the development of GBS. The most commonly used technique utilises enzyme-linked immunosorbent assays (ELISA) to measure antibody response to infection. The antigens used in these tests have been protein rich antigens that detect antibodies to common, cross-reactive epitopes and are not serotype specific (Nachamkin, Allos and Ho, 1998).

Serum IgG, IgM and IgA rise rapidly in response to *Campylobacter* infection with IgA beginning to decline during the first few weeks followed by a decline in IgM and IgG 3 to 4 weeks post infection (Blaser and Duncan, 1984). IgA has also been detected in faeces and urine of some patients but only in the first few weeks after acute infection (Lane *et al*, 1987). While detection of a fourfold rise in titre is useful to confirm a recent infection with *Campylobacter* in GBS patients, acute sera are frequently unavailable. Serological studies have therefore relied upon comparing antibody levels in GBS patients with a general population control group.

Table 1.4 summarises the previously published associations between *C. jejuni* enteritis and GBS. Serological evidence of past infection with *Campylobacter* in these studies range from 13 - 66 % (median: 33%) of patients presenting with GBS. The youngest patient, who subsequently recovered completely, was a 2 year old girl with a severe case of GBS. The

Table 1.4: Documented associations between GBS and *Campylobacter jejuni* infections

REFERENCE	CASE (C) OR SERIES (S)	AGE & SEX	% POSITIVE FOR *C. JEJUNI* INFECTION	INTERVAL B/W DIARRHEA & ONSET OF GBS (days)	CULTURE (C), SEROLOGY (S), OR BOTH (B)
Rhodes and Tattersfield, 1982	C	45/M		15	B
Molnar, Mertgola and Erkko, 1982	C	42/M		13	C
Constant, Bentley and Denman, 1983	C	34/F		>10	C
Speed, Kaldor and Cavanagh, 1984	C	16/M		10	B
Kaldor and Speed, 1984	S (n = 56)		38		S
Pryor *et al*, 1984	C	34/M 22/F		21 21	C C
Wroe and Blumhardt, 1985	C	27/M		7	C
De Bont *et al*, 1986	C	2/F		9	S
Speed *et al*, 1987	S (n = 45)		49		C
Kohler and Goldblatt, 1987	C	69/M		13	C
Ropper, 1988	S (n = 106)		4		C
Winer *et al*, 1988	S (n - 100)		14		S
Sovilla, Regli and Francioli, 1988	C	38/M 81/M 60/M		6 8 12	C C S
Clavelou *et al*, 1989	C	30/M 62/M 74/M		12 10 15	S S B
Yuki *et al*, 1990	C	25/M 83/F		7 7	S S
Gruenewald *et al*, 1991	S (n = 17)		18		S
Kuroki *et al*, 1991	C	7/M 9/M 10/F 11/M 13/M 14/M 14/M		 6 15 5 6 6 7	C C B C B C C
Boucquey *et al*, 1991	S (n = 45)		13		S
van der Meche *et al*, 1992	S (n = 129)		36		S
Gregson, Koblar and Hughes, 1993	S (n = 42)		36		S
Kuroki *et al*, 1993	S (n = 46)		41		B
Mishu *et al*, 1993	S (n = 116)		36		S
Enders *et al*, 1993	S (n = 38)		39		S
Vriesendrop *et al*, 1993	S (n = 58)		17		S
Rees *et al*, 1995	S (n = 103)		26		B
Ho *et al*, 1995	S (n = 38)		66		S
Koga *et al*, 2001	S (n=220)		17		S

M = Male, F = Female, n = number of patients. Adapted from Hughes and Rees, 1997.

oldest patient, was an 83 year old with severe axonal degeneration who remained bedridden 3 years after her illness.

With the recent attempt to separate GBS into clinical subgroups, Ho *et al* (1995) found that raised antibody levels were detected in both the AIDP and AMAN types of GBS in patients from China.

1.7.3 (b) Cultural studies of GBS

As many patients have ceased excreting *Campylobacter* in their faeces by the time symptoms of GBS develop, culture studies are not widely utilised. However, a number of well documented cases summarised in Table 1.4 clearly supports the association of a recent enteritis with *C. jejuni* and development of GBS. There was an average of 10.3 days (range 5-21) duration between the onset of gastroenteritis and the onset of neuropathic symptoms in these studies compared with the 9.5 days (range 9-12) seen in serological studies.

Many studies have been performed to determine the convalescent excretion of campylobacters following acute diarrhoeal illness. In one study of children in Thailand, the duration of excretion was found to be 14 +/- 2 days for children < 1 year old and 8 +/- 2 days for children 1-5 years old (Taylor *et al*, 1988). Similarly, in children less than 5 years of age in a Mexican study excretion was found to continue for 7 days (range 7-26)(Calva *et al*, 1988). In other studies longer excretion times have been found. In one Norwegian study, the average excretion time was 37.6 days (range 15-69)(Kapperud *et al*, 1992). Differing culture techniques, incubation temperatures, atmospheric gases and frequency of sampling all play a part in the variations found between studies therefore highlighting the need for optimum conditions when culturing for low numbers of *Campylobacter* in GBS patients.

The optimum number of samples required from patients with GBS has also been studied. It was found by Kuroki *et al* (1993) while studying 14 GBS patients, that 57% of patients grew *Campylobacter* in only the first sample tested. This increased to 93% after 2 samples were tested and all 14 patients grew *Campylobacter* after 3 samples had been tested, thus emphasising the need for multiple sampling.

1.7.3 (c) Molecular mimicry theory of GBS pathogenesis

The cell wall of *C. jejuni* has been found to contain high molecular weight smooth-form lipopolysaccharides (LPS) in one third of serotype reference strains (as is also found in the members of Enterobacteriaceae)(Preston and Penner, 1987). This consists of an O-specific polysaccharide chain, which is a polymer of repeating oligosaccharide units, a core

oligosaccharide (OS) and lipid A (Figure 1.2). The remaining two thirds of serotype reference strains have a low molecular weight LPS. Once thought to be similar to the rough form of LPS found in members of the family Enterbacteriaceae, the structure has been found to closely resemble lipo-oligosaccharides (LOS) found in *Neisseria* spp. and *Haemophilus* spp. (Aspinall *et al*, 1993).

Comparison of lipid A in different Gram negative genera reveal a similar architecture. While there are variations at the genus level in the type of hexosamine present, degree of phosphorylation, presence of phosphate substituents and most notably the nature, chain length, number and location of fatty acyl chains present, each Gram negative species has a unique type of lipid A (Moran, 1995).

The core region of LPS can be separated into two distinct regions, the inner core and the outer core. Within a bacterial species, chemical variation in this core region is greater than that of lipid A but more limited than the O-specific chain. For example, *E. coli* can be divided into five types of core regions (R1-R4 and K12) but over 100 serotypes based on the O-specific chain have been identified (Moran, 1997).

Within the core region there is greater variation, with the outer core varying more than the inner core region (as seen in Figure 1.3 which shows the variation within the core regions of GBS associated *Campylobacter* serostrains O:1, O:4, O:2, O:10). The core structure of a serostrain O:3, which has never reported to be associated with GBS is included for comparison (Moran, 1997).

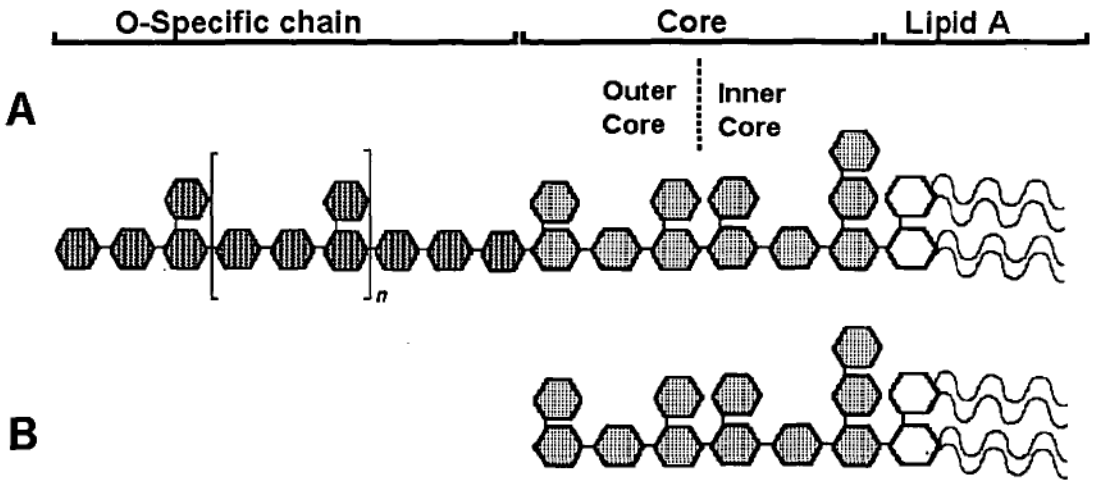
Gangliosides are sialic acid containing glycosphingolipids present in the plasma membrane of vertebrate tissues which are particularly abundant in the nervous system (representing 10% of the total lipid content).

Serum antibodies against gangliosides have been reported in 30% of GBS patients (Mishu and Blaser, 1993). Autoantibodies are frequently detected in sera of GBS patients in the acute phase of illness, especially Gm1 but other antibody specificities detected are, GD1b, GT1a, GQ1b and LM1, as well as but with lower frequency asialoGM1, GM2, GM3, GD1a and GT1b (Schwerer *et al*, 1995).

It was first demonstrated by Yuki *et al* (1993), that the antibodies produced against a commonly encountered GBS associated serotype of *C. jejuni* (O:19), cross reacted with Gm1 gangliosides. Further investigations have found similarities in core structure of this serotype to gangliosides Gm1, GD1a, GT1a and GD3 (Aspinall *et al*, 1994a).

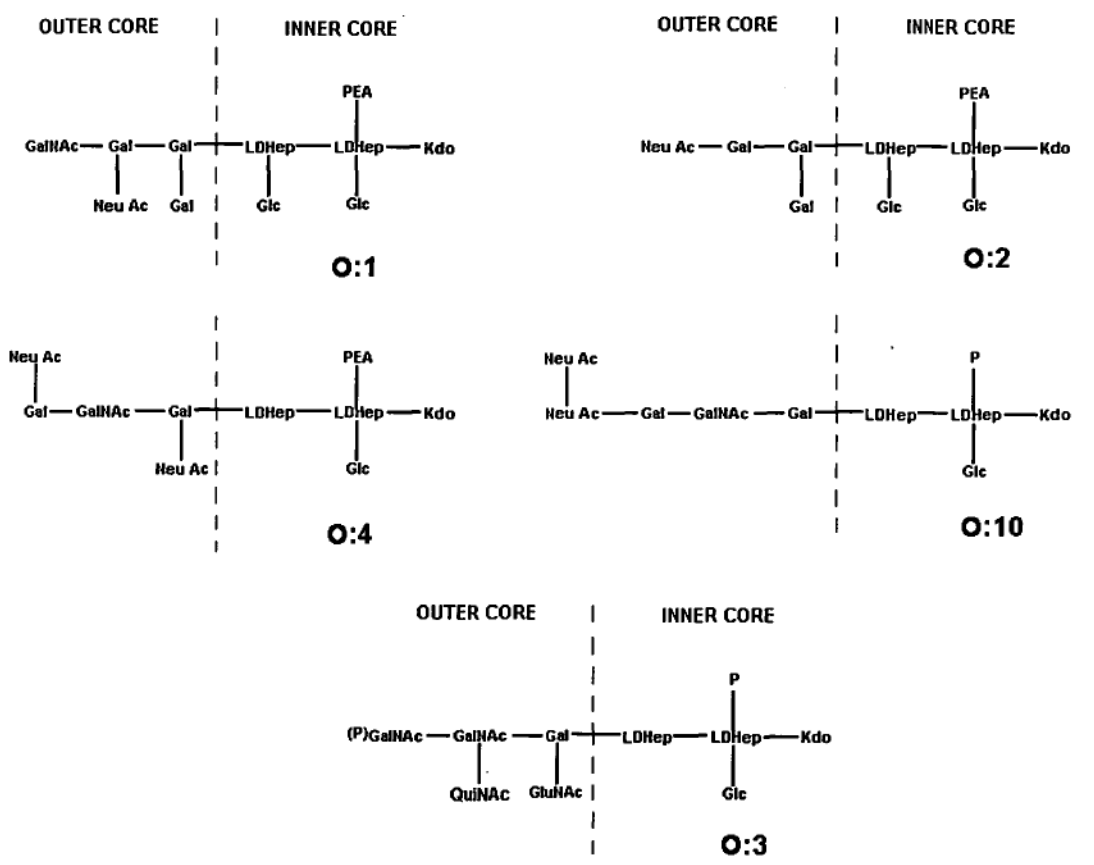
Mimicry of gangliosides by core OS of *C. jejuni* LPS, as well as the relationship between *C. jejuni* infection and induction of ganglioside-antibodies in GBS, suggest that LPS from certain strains could act as cross-reactive antigens for anti-ganglioside B cells. Furthermore,

Figure 1.2: Schematic representation of the architecture of *C. jejuni* cell wall : A) high molecular weight LPS and B) low molecular weight LOS



Adapted from Moran, 1997.

Figure 1.3: Core structure of *C. jejuni* serostrains.



Glc: D-glucose, Gal: D-galactose, P: O-phosphoryl group, PEA: O-phosphoethanolamine, Kdo: 3-deoxy-D-manno-2-octulosonic acid, LDHep: L-glycero-D-manno-heptose, QuiNAc: N-acetyl-D-quinovosamine, GalNAc: N-acetyl-D-galactoseamine, GluNAc: N-acetyl-D-glucosamine, NeuAc: N-acetylneuroaminic acid
Adapted from Moran, 1997.

laboratory animals injected with *C. jejuni* LPS have been found to produce auto-antibodies against gangliosides (Ritter *et al*, 1996).

Other serotypes associated with GBS have also been found to contain an outer core region with similarities to gangliosides. For example, the terminal pentasaccharide of serostrain O:4 mimics GD 1a and the termini of core OS in O:23 and O:36 mimic the same tetrasaccahride present in GM2 (Aspinall *et al*, 1993). The same similarities have been noted in strains from MFS, ie. a *C. jejuni* O:10 strain associated with MFS possessed core OS structure that mimics ganglioside GD3 (Salloway *et al*, 1996). Figure 1.4 demonstrates the similarities found between human ganglioside Gm1 and the two types of core structures found in serostrain O:19, one of *Campylobacter* serostrains frequently associated with GBS.

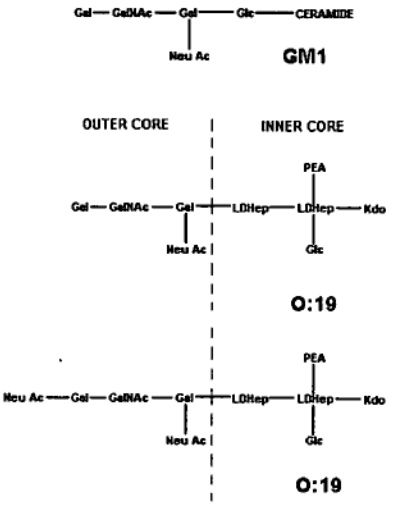
1.8 Summary

Numerous culture and serological studies have suggested that *C. jejuni* is the infectious agent most commonly associated with the development of GBS. With *Campylobacter* infection being the most common cause of bacterial food poisoning in the Western world, it is somewhat surprising that such a small number of patients progress to develop this neurological condition. Two possibilities exist; the first being that susceptibility is determined by host specific factors; the second is that strain-specific factors determine whether patients develop GBS.

Evidence has emerged recently that suggests certain *C. jejuni* strains possess structures in their cell wall lipopolysaccharides that mimic gangliosides, particularly Gm1, as found in vertebrate nervous tissue. Infections with these strains elicit an autoimmune response in GBS patients with serum antibodies to these gangliosides being reported in many cases. While many serotypes possess these structures, certain serotypes, in particular Penner serotype O:19, appear to be over represented in most studies of GBS. It should be noted that not all serotype O:19 *C. jejuni* infections progress to GBS, not all GBS patients with a prior *Campylobacter* infection have detectable levels of anti-ganglioside antibodies in their serum and up to one third of all strains isolated from cases of gastroenteritis possess Gm1 like epitopes in their bacterial cell walls but do not progress onto GBS. Further subtyping of these strains is therefore needed to identify other bacterial factors that may contribute to their neuropathogenic nature.

The aim of this investigation was to obtain an overall view of *Campylobacter* infection in Tasmania comparing prevalence rates of GBS with cases of *Campylobacter* gastroenteritis. Full speciation of isolates was performed and characterisation of the known GBS associated markers, Gm1 and serotype O:19, was determined to allow potential neuropathogenic strains

Figure 1.4: The structure of human ganglioside Gm1 and *Campylobacter jejuni* serostrain 0:19 cell wall core region.



Glc: D-glucose, Gal: D-galactose, PEA: O-phosphoethanolamine, Kdo: 3-deoxy-D-manno-2-octulosonic acid, LDHep: L-glycero-D-manno-heptose, GalNAc: N-acetyl-D-galactoseamine, NeuAc: N-acetylneuroaminic acid
Adapted from Moran, Appelmelk and Aspinall, 1996.

to be identified. Furthermore, specific bacterial markers were sought by using Pulsed-Field Gel Electrophoresis (PFGE) and Subtractive Hybridisation to highlight regions of the *Campylobacter* genome of interest. Finally, production of a more accurate and rapid Polymerase Chain Reaction (PCR) assay has been developed for the detection of those strains which trigger the autoimmune response seen in GBS.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Equipment

All materials and equipment used in this study along with the suppliers from which they were obtained are listed in Tables 2.1 and 2.2 respectively. All chemicals used were analytical reagent grade. All solutions were stored and used at room temperature unless otherwise stated.

Table 2.1: Reagents and suppliers

MATERIALS	SUPPLIERS
Acetic acid	BDH Laboratory Supplies, England Cat. No.: 100015N
Acrylamide	Promega Cat. No.: 32179
AdvanTage™ cDNA PCR Kit	Clontech, CA., USA. Cat. No.: K1905-Y
AdvanTage™ PCR Cloning Kit	Clontech, CA., USA. Cat. No.: K1901-1
Agarose NA	Pharmacia Biotech, Sweden Cat. No.: 17055402
Agarose Prep. (LMP)	Pharmacia Biotech, Sweden Cat. No.: 80113007
Ammonium acetate	ICN Biomedicals, Sydney, Aust. Cat. No.: 100651-17
Ammonium persulphate	Biorad Cat. No.: 161-0700
Amplitaq Gold® DNA Polymerase with GeneAmp	Roche Molecular Systems Inc., USA Part No. N808-0243
Anti-*Cholera* Toxin (Rabbit)	Sigma Chemical Co., USA Cat. No.: C3062
Anti-Digoxigenin-alkaline phosphatase conjugate	Boehringer Mannheim, Germany Cat. No.: 1093274
Antimicrobial susceptibility disc Cephalothin 30 ug	Oxoid, UK. Cat.No.: A KF30
Antimicrobial susceptibility disc Nalidixic acid 30 ug	Oxoid, UK. Cat. No.: A NA30
Anti-Rabbit IgG (Whole Molecule) Peroxidase Conjugate	Sigma Chemical Co., USA Cat. No.: A9169
API Campy	bioMerieux, France. Cat. No.: 20800
Bacto-tryptone	Difco Laboratories, USA Cat. No.: 211705
BigDye™ Terminator v3.0 Cycle Sequencing Read Reaction Kit	ABI Prism, Applied Biosystems Cat. No.: 4390242
Bisacrylamide	Promega Cat. No.: V3143

MATERIALS	SUPPLIERS
Biotinylated probe synthesis	Biosearch Technologies, Inc., Australia
Blocking Reagent	Boehringer Mannheim, Germany Cat. No.: 1096176
Blue-White Select™ Screening Reagent (X-gal/IPTG)	Sigma Chemical Co., USA Cat. No.: B3928
Boric Acid	Merck, Germany Cat. No.: 1.12015.1000
Bromophenol Blue	International Biotechnologies Inc, USA Cat. No.: 74040
Campylobacter Agar	Oxoid, UK. Cat. No.: CM 739
Campylobacter Antibiotic Supplement	Oxoid, UK. Cat. No.: SR 174
Cholera Toxin B Subunit (Choleragenoid)	Sigma Chemical Co., USA Cat. No.: C9903
Chloroform	ICN Biomedicals, Sydney, Aust. Cat. No.: 510710
Columbia Blood Agar supplemented with Horse Blood, NAD and Haematin	Oxoid, UK. Cat. No.: CM331
Coomassie brilliant Blue – R250	ICN Biomedicals, Sydney, Aust. Cat. No.: 190682
CSPD	Boehringer Mannheim, Germany Cat. No.: 1755633
dATP, dCTP, dGTP, dTTP (100 nM)	Promega Corporation, USA Cat. No.: U1240
DIG DNA labelling kit	Roche Molecular Systems Inc., USA Cat No. 1175037
DyeEx™ Spin Kit	QIAGEN Cat. No.: 63206
EDTA, di-Sodium Salt	International Biotechnologies Inc, USA Cat. No.: 70182
Ethanol (ethyl alcohol)	BDH Laboratory Supplies, England Cat. No.: 10107.7Y
Ethidium Bromide	Sigma Chemical Co., USA Cat. No.: 8751
Ficoll	ICN Biomedicals, Sydney, Aust. Cat. 194824
Gas Generating Kit	Oxoid, UK. Cat. No.: BR056A
Glycine	ICN Biomedicals, Sydney, Aust. Cat. 808822
Gm1 monosialoganglioside 1mg	Sigma Chemical Co., USA Cat. No.: G7641
Hybond™ -N+ nylon nucleic acid transfer membrane	Amersham Pharmacia Biotech, Sweden Cat No.: 203B

MATERIALS	SUPPLIERS
Hippuric acid (Sodium salt)	Sigma Chemical Co., USA Cat. No.: H9380
iso-Amyl alcohol	BDH Laboratory Supplies, England. Cat. No.: 100383L
Kanamycin monosulfate	Sigma Chemical Co., USA Cat. No.: K4000
Lambda DNA-PFGE markers	Boehringer Mannheim, Germany Cat. No.: 1378961
Laemmli Sample Buffer	Biorad Cat. No.: 161-0737
Lysed blood sensitivity agar	Oxoid, UK. Cat. No.: CM409B
Maleic Acid	ICN Biomedicals, Sydney, Aust. Cat. No.: 102233
2-Mercaptoethanol	ICN Biomedicals, Sydney, Aust. Cat. No.: 190242
Methanol	BDH Laboratory Supplies, England. Cat. No.: 10158.4W
Microcentrifuge Polypropylene Tubes (Eppendorf) 1.5 mL	Robbins Scientific Corp., USA Part No.: 1012-00-0
Microcentrifuge Polypropylene Tubes (Eppendorf) 0.6 mL	Quality Scientific Plastics, USA Cat. No.: 502-PLN
Microtubes & Caps with O Ring 1.5 mL Screw Cap	Quality Scientific Plastics, USA Cat. No.: 515
Microtitre trays: NUNC-Immuno Plate	Nalge Nunc International Cat. No.: 442404
Mineral Oil	ICN Biomedicals, Sydney, Aust. Cat. No.: 151694
Nichrome wire (Nikrothal 80)	Kanthal Pty. Ltd., Aus
Ninhydrin	Sigma Chemical Co., USA. Cat.No.: N4876
N-laurylsarcosine, Sodium salt	ICN Biomedicals Inc, USA Cat. No.: 190289.17
Oligonucleotide synthesis	GeneWorks Pty. Ltd, Australia
OPD Peroxidase Substrate Tablet Set SIGMA FAST™	Sigma Chemical Co., USA. Cat. No.: P-9187
Parafilm	American National Can.
PCR-Select Bacterial Genome Subtraction Kit	Clontech, CA, USA. Cat. No.: K1809-1
Phenol:chloroform:isoamyl alcohol (25:24:1)	Life Technologies, GIBCOBRL, USA. Cat. No.: 15593-031
Phosphate Buffered Saline	Oxoid, UK. Cat. No.: BR14a
Polaroid Black and White Film (665)	Polaroid, USA
Polyamp Duofit™ Water for Injections BP	ASTRA Pharmaceuticals Pty. Ltd., NSW, Australia

MATERIALS	SUPPLIERS
Proteinase K	AMRESCO. Cat. No.: E634
pUC-19 DNA/*Hpa*II Molecular weight marker (100 ug/200 uL)	Bresatec Ltd., Adelaide, Australia Cat. No.: DMW-P1
QIAamp Tissue Kit	QIAGEN Cat. No.: 29306
QIAexpress UA Cloning Kit	QIAGEN Cat. No.: 32179
QIAquick PCR Purification Kit	QIAGEN Cat. No.: 28106
Restriction Endonuclease *Alu*I	Boehringer Mannheim, Germany Cat. No.: 10239275
Restriction Endonuclease *Sal*I	Boehringer Mannheim, Germany Cat. No.: 348783
Restriction Endonuclease *Sma*I	Boehringer Mannheim, Germany Cat. No.: 220566
Saline, Sterile 2.5 mls	Oxoid, UK. Cat.No.: OXTM931
Skim Milk Powder	Diploma, Australia
Silver Stain Plus	Biorad Cat. No.: 161-0449
Sodium Chloride	Sigma Chemical Co., USA Cat. No.: S9888
Sodium Citrate	BDH Laboratory Supplies, England. Cat. No.: 10242
Sodium Dodecyl Sulfate (SDS)	Sigma Chemical Co., USA Cat. No.: L4390
Strepavidin-alkaline phosphatase Conjugate 150U	Boehringer Mannheim, Germany Cat. No.: 1093266
Storage vials for bacteria (Microbank)	Pro-Lab Diagnostics, Canada Cat. No.: PL160
Sulfuric Acid	BDH Laboratory Supplies, England. Cat. No.: 7034825
SuRE/Cut Buffer A	Boehringer Mannheim, Germany Cat. No.: 1417959
SuRE/Cut Buffer H	Boehringer Mannheim, Germany Cat. No.: 1417991
TEMED – tetramethylenediamine	Biorad Cat. No. 1610801
Tris-base	ICN Biomedicals Inc, USA Cat. No.: 103132
Tris-Cl	International Biotechnologies Inc, USA Cat. No.: 70162
Xylene cyanol	ICN Biomedicals Inc, USA Cat. No.: 806801
Yeast extract	Oxoid, UK. Cat No.: L21

Table 2.2: Equipment and suppliers

EQUIPMENT	SUPPLIERS
Anaerobic jar, 3.4 litre	Oxoid, UK. Cat. No.: HP11
Anaerobic jar catalyst	Oxoid, UK. Cat. No.: BR42
Bench waterbath	Mgw Lauda Model No.: C36D
Biological Safety Cabinet Class II	CLYDE-APAC Model No: BH 2000 Series
Centrifuge (Microfuge)	Eppendorf Cat. No.: 5415C
Colorimeter (Vitek)	HACH Company, USA. Prod. No.: 52-1210
Dynatech MR5000 Spectrophotometer	Dynatech, Guernsey, Channel Islands Cat. No.: DL 1000
Dualwave Microwave Oven	NEC Model No.: N513M
Finnpipette® Pipettes	Labsystems
Freezer – Ultra Low (- 80°C)	SANYO Model. No.: MDF-U4086S
Gene Navigator Control Unit	Amersham Pharmacia Biotech, Sweden Part No.: 18-1026-17
Gene Navigator Electrophoresis Unit	Amersham Pharmacia Biotech, Sweden Part No.: 18-1019-20
Gene Power Supply	Amersham Pharmacia Biotech, Sweden Model No.: GPS 200/400
Hybridisation Incubator	Robbins Scientific Model No.: 2000
Hypercassette™	Amersham Pharmacia Biotech, UK Cat. No.: 11643
Hyperfilm™ -MP Autoradiography film	Amersham Pharmacia Biotech, UK Cat. No.: 3103K
MINIPROTEAN®3 Cell Electrophoresis Equipment	Biorad Cat. No.: 525BR
Photoautomat Wild MPS 46/52	Wild Leitz, Ltd., Switzerland
Polaroid MP-4 Land Camera	Polaroid, USA
Perkin Elmer DNA Thermal Cycler	Perkin Elmer Model No.: 9600
Perkin Elmer DNA Thermal Cycler	Perkin Elmer Model No.: 480
RFLP Main & Advanced Computer Program	American Applied Biotechnology, USA
Spectophotometer : Ultraspec II	Amersham Pharmacia Biotech, Sweden Prod. No.: 80-2097-62
UV Transilluminator	UVP Inc., USA Model No.: TM36

2.2 Sample Collection and Storage

Campylobacter isolates were collected from the Microbiology departments of the five major pathology providers in Tasmania which are;

i) Royal Hobart Hospital
ii) Launceston General Hospital
iii) Hobart Pathology
iv) Launceston Pathology
v) North West Pathology

In total, 249 isolates were obtained from faecal samples from patients presenting with gastroenteritis and one isolate was from blood cultures.
All faecal strains were originally isolated on Oxoid *Campylobacter* media, with antibiotic supplement CAT SR174 added, after being incubated at 42°C in an Oxoid 3.4 litre anaerobic jar with catalyst. *Campylobacter* gas generating kits were used to generate a microaerophilic atmosphere.
All isolates were subcultured onto horse blood agar (HBA) on arrival at the testing laboratory and incubated for 48 hours at 42°C in a microaerophilic atmosphere. The total growth on the agar plate was then removed with a sterile swab and inoculated into a Pro-Lab Diagnostics Microbank storage tube. These were then stored - 80°C.

2.3 Bacterial Culture

2.3.1 *Campylobacter* species

All *Campylobacter* species were subcultured onto horse blood agar from the Microbank storage tubes held at - 80°C and grown at 42°C for 48 hours in an Oxoid 3.4 litre anaerobic jar with an atmosphere generated by the Oxoid *Campylobacter* gas generating kit. Poor growing strains were subcultured and regrown at 37°C under the same atmospheric conditions. Repeated subculturing was kept to a minimum and experiments were always performed on fresh subcultures from the original - 80°C stock culture.

2.3.2 *Escherichia coli*

Escherichia coli used in cloning experiments were grown on LB/X-gal/IPTG agarose plates containing 50 μg/mL of kanamycin (as prepared in Appendix B). Transformed *Escherichia coli* were grown overnight at 37°C in aerobic atmospheric conditions.

2.4 *Campylobacter* Biochemical Identification

2.4.1 Hippurate Hydrolysis

All isolates were tested for hippurate hydrolysis as described by Hwang and Ederer, (1975). This was performed by adding a loopful of organism to 0.4 mL of sodium hippurate in phosphate buffer (pH 7.0). This suspension was incubated at 37°C for 2 hours and 0.2 mL of 3.5% ninhydrin added. The colour change was then read following a 10 minute incubation at 37°C. Positive reactions were indicated by a strong violet colour, with clear or pale violet recorded as a negative reaction. A control strain of *C. jejuni* and *C. coli* was included during testing as positive and negative controls respectively.

2.4.2 Sensitivity Testing

Sensitivity testing of isolates was performed against nalidixic acid (30 μg) and cephalothin (30 μg) antibiotic disks using the CDS method (Bell, 1975). A 0.56 mm diameter nichrome wire was stabbed into a single colony of the organism to be tested and the wire then used to inoculate a 2.5 mL sterile saline solution. The saline solution was then poured onto a lysed sensitest agar and poured off. The agar plate was allowed to dry at RT and the two antimicrobial discs placed onto the agar surface. Following incubation overnight at 42°C in a microaerophilic atmosphere produced by Oxoid *Campylobacter* gas generating kits, the radius of growth inhibition was recorded. Organisms were reported sensitive if the radius of growth inhibition was > 6 mm and resistant if < 6 mm.

All isolates with a positive hippurate test and which were sensitive to nalidixic acid and resistant to cephalothin were designated *C. jejuni* subsp. *jejuni* (Cowan and Steel, 1993).

2.4.3 Extended Biochemical Testing

All isolates not identified as *C. jejuni* subsp. *jejuni* were further identified by the commercially available identification kit, API Campy (bioMerieux). Fresh overnight cultures of bacteria were used to inoculate prepackaged biochemical strips followed by appropriate incubation as per kit instructions. Following reading of test results at 48 hours, an octal code number generated by the test results was compared to the API database program and a percentage probability of identification produced.

2.5 Nucleic Acid Extractions

The Qiagen QIAamp® Tissue DNA extraction kit was used to purify bacterial DNA. The "Bacterial Protocol" as outlined in the QIAamp® Blood and Tissue Kit Handbook was performed without modification.

2.6 Polymerase Chain Reaction

2.6.1 Laboratory Practices

Appropriate laboratory practices were employed at all times during testing as outlined by standard molecular biology procedures to reduce the risk of carryover contamination. Laboratory coats were worn at all times in designated laboratory areas and disposable gloves used when handling all reagents and apparatus. Materials used, such as pipette tips and microfuge tubes were sterilised in sealed autoclave bags prior to use by standard autoclave procedures for 15 minutes at 121°C. Prevention of contamination was achieved by using plugged pipette tips at all times and changing pipette tip between the addition of each reagent to prevent cross-contamination of reagents. Reagents were stored in aliquots of working volume concentrations at –20°C and thawed just prior to use at room temperature.

Polymerase Chain Reaction assays were performed in a physical containment class 2 (PC2) laboratory located within the Pathology Department of the Royal Hobart Hospital. The laboratory is divided into three separate rooms with interconnecting portals for one way transference of prepared reagents for contamination minimisation. Room one was designed for preparation of reagents used in PCR which are free of nucleic acid. Prepared reagents were passed through the portal into the second room. DNA extraction and inoculation into prepared PCR reagents occurred in Class II biological safety cabinets in room two and was amplicon free. PCR amplification was performed on Perkin Elmer DNA Thermal Cyclers followed by product detection by gel electrophoresis in room three.

2.6.2 Primer Selection

Primer selection for the serotype O:19 specific PCR (DNA gyrase subunit B gene) was chosen from the literature as outlined in Table 2.3. All other primers were designed in this study using gene sequences obtained from Genbank using the nucleotide search engine at the National Centre for Biotechnology Information (NCBI) web page. (http://www.ncbi.nlm.nih.gov)

Designed primers were compared for specificity against the Genbank database using the computer program Basic BLAST also accessed at the NCBI site.

Table 2.3: Details of primers chosen and designed

Primer Name	Length	Amplified Region	Sequence (5' to 3')	Product Size	Reference
Hippurate Sense	23 n.t.	Hippurate gene GenBank: cjhippurc (1554– 1931 b.p.)	GGACTTCGTGCAG ATATGGATGC	377 b.p.	This study
Hippurate Antisense	23 n.t.		GCACTTCCATGAC CACCTCTTCC		
16S rRNA Sense	23 n.t.	16S rRNA gene GenBank: Campylobacter & Arcobacter species	GGAGGATGACAC T/ATTTCGGT/AGC G	842 b.p.	This Study
16S rRNA Antisense	28 n.t.		TCA/GCC/GGTATT GCT/GT/GCTCT/AT TGTATAC/TC/GC		
GyrB Sense	18 n.t.	DNA gyrase subunit B (1222– 1673 b.p.)	CAAGCTATACTGC CTTTG	451 b.p.	Misawa, Allos and Blaser, 1998
GyrB Antisense (O:19)	18 n.t.		TCAAGATCTTTTA AAATT		
GyrB Antisense (Non O:19)	18 n.t.		TCAAGATCTTTTA AAATC		
3.21 Sense	17 n.t.		GAT CGC TAC GCA GGT TC	162 b.p.	This study
3.21 Antisense	18 n.t.		GGT GCC TTG TTC GTC TTG		
Primer 1	22 n.t.	Adaptor 1 and 2R Subtractive Hybridisation Kit	CTAATACGACTCA CTATAGGGC	Variable	CLONTECH Laboratories Inc.
Nested Primer 1	22 n.t.		TCGAGCGGCCGCC CGGGCAGGT		
Nested Primer 2R	20 n.t.		AGCGTGGTCGCGG CCGAGGT		

Primer Name	Length	Amplified Region	Sequence (5' to 3')	Product Size	Reference
Veh18 Sense	**18 n.t.**		**AATNCTATCATCA AAATG**	**569 b.p**	
Veh19 Sense	**19 n.t.**		**CAATNCTATCATC AAAATG**	**570 b.p**	**This Study**
Veh20 Sense	**20 n.t.**	***Veh* gene**	**GCAATNCTATCAT CAAAATG**	**571 b.p**	
VehRev Antisense	**20 n.t.**		**GAGGTCTTTAAGG TGTTTTC**		

n.t. = nucleotides, b.p. = base pairs

2.6.3 Product Controls

All PCR tests were performed with DNA extracted from appropriate standard strains of *C. jejuni* for a positive control and water as a negative control.

2.6.4 Product Detection

Amplified DNA was detected using agarose gel electrophoresis. PCR amplified nucleic acid samples were mixed with 1/10 volume of loading buffer (final concentrations: 10mM Tris-HCl, pH 8.0; 1 mM EDTA; 3% (w/v) ficoll; 0.05% bromophenol blue; 0.05% xylene cyanol). 20.0 µL samples were then loaded into 10mm wells of a 3% molecular biology grade agarose gel in 1 X TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA). The staining agent, ethidium bromide, was incorporated into the gel at a concentration of 1.0 µg/mL. Each electrophoresis run included the molecular weight marker (pUC-19 restricted with *Hpa*II).

The products were separated by horizontal electrophoresis in 1 X TAE buffer at 100 V for 60 minutes. Visualisation of the separated products was achieved using a UV transilluminator and the results photographed using a Polaroid MP-4 land camera with Polaroid type 665 film. A positive result was defined as the presence of a band of the appropriate molecular weight.

2.7 Cholera **Binding Inhibition EIA For The Detection Of Gm1 Epitopes On Bacterial Cell Walls**

This method was a modification of that of Sack *et al*, 1998.

2.7.1 Preparation of test *Campylobacter*

Bacteria were grown on Horse Blood Agar (HBA) for 24 hours in a gas pack jar (Oxoid) with Campy gas packs (Oxoid) at 42°C. The bacteria were harvested into 2 ml of Phosphate Buffered Saline (PBS) (pH 7.2, 0.01M) and transferred to a test tube. The concentration of the bacteria was then adjusted with PBS until a transmission equaling 10% on a Vitek colorimeter was obtained. This solution was then transferred into a sterile 1.5 ml Eppendorf tube and placed into a boiling water bath for 15 minutes.

The suspension was then held at 4°C until testing was performed. Bacterial lipopolysaccharides (LPS) are extremely stable under these conditions and can be stored there for extended periods of time.

2.7.2 Preabsorption of Cholera B subunit to Bacterial LPS

Plates were prepared for testing by placing 100 µL of the boiled bacterial suspensions (2.7.1) into wells of a microtitre plate followed by 100 µL of Cholera B subunit (0.2 µg/ml, diluted with PBS). The tray was covered with parafilm and incubated at 37°C for 1 hour. Following incubation, the OD at 490 nm of the suspension was determined on a Dyntech MR5000 spectrophotometer and the test carried out only if sufficient bacteria were present, as indicated by an OD of greater than 0.1.

2.7.3 Testing mictotitre tray

A microtitre tray was coated with Gm1 ganglioside (Sigma) (1µg/mL in PBS) at 4°C for 24 hours and then washed with 3 changes of PBS for 30 seconds each. The trays were blocked with 200 µL of 5% skim milk in PBS at RT for 1 hour. Following 3 washes with PBS for 30 seconds, 100 µL of suspension from the preparation tray was transferred to the corresponding testing tray well, covered with parafilm and incubated at 37°C for 2 hours. The tray was washed as described above and rabbit anti-Cholera toxin (Sigma) (diluted 1/1250 in PBS) was added to each well, covered in parafilm and incubated at 37°C for 1 hour. The tray was washed as described above and anti-rabbit peroxidase conjugate (Sigma) (diluted 1/1250 in PBS) was added to each well, covered with parafilm and incubated at 37°C for 1 hour. The tray was then washed as described previously and 100 µL of substrate added to each well (Sigma Fast – OPD) prior to incubation at RT for 10 minutes in the dark. The colour development was stopped by the addition of 100 µL 3M H_2SO_4 and the OD read at 490 nm.

A *C. jejuni* Gm1 positive strain (ATCC 700297) and *C. jejuni* Gm1 negative strain (ATCC 43431) were included in each assay batch. Positive strains inhibit the development of colour and are defined as strains which inhibit the OD by >50% relative to the negative control.

2.8 Dot Blot Hybridisation

Dot blot hybridisation was performed in two separate experiments. Firstly, biotinylated 16S rRNA probes were used to identify *Campylobacter* species with strepavidin-alkaline phosphatase as the conjugate. Secondly, digoxigenin labeled probes were used in cloning experiments using anti-digoxigenin-alkaline phosphatase as the conjugate.

2.8.1 DNA Preparation

Details of the reagents used in the dot blot hybridisation assay are supplied in appendix C. DNA samples were first denatured by heating at 95°C for 10 minutes. A 4 µL sample was spotted onto a labeled segment of Hybond™ -N+ nylon nucleic acid transfer membrane along with positive and negative control strains and bound by exposing to UV on a UV transilluminator for one minute on both sides of the membrane.

2.8.2 Probe preparation

In-house digoxigenin labeling of DNA was performed using the DIG DNA Labeling Kit - Boehringer Mannheim according to manufacturers instructions. Biotinylated probes were synthesized by Biosearch Technologies, Inc., Australia.

2.8.3 Hybridisation

The membrane was placed into prehybridisation buffer and incubated in a hybridisation oven at a temperature appropriate for the probe being tested. This was then incubated for 1 hour, followed by overnight incubation in prehybridisation buffer containing the appropriate probe.
The membrane was washed twice in wash solution 1 for 5 minutes at room temperature, followed by two 15 minute washes in wash solution 2 at the same temperature used for the probe incubation. Two final washes were given in solution 3 for 5 minutes at room temperature. The membrane was then blocked in blocking solution for 30 minutes at room temperature followed by incubation with the conjugate in blocking solution for 15 minutes at room temperature. Two 15 minute washes in wash solution 3 were performed at room temperature before a final wash in detection buffer for 5 minutes at room temperature.

2.8.4 Detection

Membranes were then incubated in 2 mLs of detection buffer containing the substrate CSPD (1:100 dilution of stock) for 5 minutes at room temperature and blotted briefly on blotting paper before being sealed in a plastic bag and incubated at 37°C for 15 minutes. The membrane was next placed in a Hypercassette™ and exposed to Hyperfilm™ -MP autoradiography film for 1 hour and processed by a Wild Leitz, Photoautomat Wild MPS 46/52 developer in the Medical Imaging department of the RHH. A positive reaction was defined as the presence of a black spot present on the x-ray film similar in intensity to the positive control strain.

2.9 Pulsed-Field Gel Electrophoresis

2.9.1 Preparation and standardisation of DNA

Details of the reagents used for pulsed-field gel electrophoresis are supplied in Appendix D. Preparation of *Campylobacter* DNA and subsequent restriction endonuclease digests were adapted from the original method by Smith and Cantor, 1987.
Campylobacters were grown overnight on a HBA plate and the active growth was removed using a sterile swab and placed into 10 mLs of PIV solution until a transmission of 70% (equivalent to a MacFarland 1.0 standard) was achieved, as determined by a Vitek colorimeter. This suspension was then transferred to a centrifuge tube and spun at 540 g for 10 minutes. The supernatant was discarded and the deposit resuspended in 300 µL of PIV solution. An equal volume of molten 2.4 % LMP agarose was then added and the subsequent mixture dispensed into block moulds and allowed to set for 15 minutes.

2.9.2 Bacterial cell disruption and protein degradation

Bacterial agar plugs were pushed out of the block moulds into 300 µL of ESP solution in a sterile Eppendorf tube and incubated in a 56°C waterbath overnight. The agarose block was then removed from the tube, washed 6 times at room temperature in 10 mls volume of TE1 solution over a 6 hour period.

2.9.3 Endonuclease digestion

Agarose blocks were cut in half and were are equilibrated in 2, 30 minute washes in 400 µL of the appropriate restriction buffer. (SuRe/Cut Buffer A for *Sma*I and SuRE/Cut Buffer H for *Sal*I). This solution was replaced with 25 µL of restriction buffer containing 30 to 40 units of

restriction endonuclease and incubated at the appropriate temperature overnight (*Sma*I incubated at 26°C and *Sal*I incubated at 37°C).

2.9.4 Electrophoresis

Quarter blocks containing 90 µg of intact genomic DNA were loaded into 5mm x 1mm wells of a 1.2% agarose gel.(Agarose NA, Pharmacia Biotech). Each electrophoresis run included a molecular weight marker (Lambda DNA: PFGE Marker 1: Boehringer Mannheim). Electrophoresis was performed at 170 V on a Gene Navigator system (Amersham Pharmacia Biotech) in three phases; 5 second pulse times for 10 hours, 20 second pulse times for 10 hours and 80 second pulse times for 4 hours.
All electrophoresis was carried out in half strength TBE at 8.0°C. Gels were then stained in ethidium bromide (0.5 µg/mL in 1X TBE) for 30 minutes and RFLP patterns visualised on a UV transilluminator and the results photographed. DNA fragments ranging in size from 50 kb to 500 kb were typically seen and easily separated using this procedure.

2.9.5 Analysis

RFLP software package from AAB (Advanced American Biotechnology) was used to detect and calculate molecular weights of individual bands seen on electrophoresis gels. Patterns of type strains were also stored in its database for comparison of new strains and dendrograms produced to show relative similarities between strains.
By using the unweighted-pair group method with arithmetic averages (UPGMA) using simple band match and a tolerance of 3.2%, dendrograms were produced to demonstrate the relationship between Tasmanian strains and to compare with PFGE profiles of GBS related strains from around the world.

2.10 Subtractive Hybridisation

2.10.1 DNA subtraction

The CLONTECH PCR-Select™ Bacterial Genome Subtraction Kit was used according to the kit instructions with the following modifications. The tester DNA used was extracted from a GBS associated *Campylobacter jejuni* (ATCC 700297: Serotype O:19) and the reference driver DNA from a *Campylobacter jejuni* serotype not associated with GBS (ATCC 43431: Serotype O:3). Also the restriction endonuclease supplied with the kit was replaced with *Alu*I.

2.10.2 Cloning

Cloning of the subtractive library produced from the subtractive hybridisation experiment was performed with the pT-Adv 3.9 kb cloning vector using the AdvanTAge™ PCR Cloning Kit from CLONTECH Laboratories, Inc. according to the kit instructions.
The expression vector pQE 30 UA was used on clone 3.21 according to QIAGEN QIAexpress UA Cloning Kit instructions.

2.11 SDS-Polyacrylamide Gel Electrophoresis

Bacterial whole cell protein profiles were produced by SDS-PAGE according to the method of Hames (1990).

2.11.1 Gel Preparation

Polyacrylamide gels containing a 5% stacking gel and a 15% resolving gel were prepared from a stock solution of 30% acrylamide and 0.8% bisacrylamide. The stacking gel was prepared in 0.125M Tris-HCl, pH 6.8, 0.1% SDS, 0.1% tetramethylenediamine (TEMED) and 0.0525M ammonium persulphate (APS). The resolving gel was prepared in 0.375M Tris-HCl, pH 8.8, 0.1% SDS, 0.0625% TEMED and 0.034% APS.
MINIPROTEAN® 3 Cell Electrophoresis Equipment (Biorad) was used with the gel forming apparatus supplied with the kit assembled according to the manufacturers instructions. Resolving and stacking gels of 10 cm and 1.5 cm respectively were poured and allowed to polymerize over an hour.

2.11.2 Sample preparation

Biorad's Laemmli sample buffer was used. This was prepared by adding 50 µL 2-mercaptoethanol to 950 µL of sample buffer. This in turn was used to dilute bacterial samples 1:2 and the sample was then heated in a boiling water bath for 3 minutes. Once cooled, 10 µL of samples were then loaded per electrophoresis track.

2.11.3 Electrophoresis

Electrophoresis was performed with a reservoir buffer containing 0.025M Tris, 0.192M glycine and 0.1% SDS (pH 8.3) at 150 volts until the dye linked proteins had migrated within 2 mm of

the bottom of the gel. Dye linked protein standards were used to monitor running conditions and to calculate approximate molecular weights of resolved sample proteins.

2.11.4 Staining

Proteins were visualised by staining with Coomassie Brilliant Blue (0.1%) which was dissolved in water:methanol:glacial acetic acid (5:5:2 by volume) for 2 hours. Excess stain was then removed and destaining performed with a one hour wash with 30% methanol, 10% acetic acid followed by multiple washes with 7 % acetic acid alone until background stain was completely removed.

For the detection of proteins in low concentrations within complex protein samples, Biorad's Silver Stain Plus was used according to manufacturers instructions.

2.12 DNA sequencing

2.12.1 Purification of PCR products

Purified PCR product for sequencing was prepared using the QIAquick™ PCR purification (Qiagen) as described in the kit instructions without modification.

2.12.2 DNA Sequencing Reactions

All sequencing primers are listed in Table 2.3: Details of primers chosen and designed, and throughout the text. Purified PCR templates were sequenced using the BigDye™ Terminator v3.0 cycle sequencing reaction as described in the kit manual without changes to the protocol. The linear amplification of DNA for sequencing was performed in a Perkin Elmer 9600 DNA Thermal Cycler. Excess dye-terminator was removed with the DyeEx™ 2.0 Spin Kit according to kit instructions. Samples were dried and sent to Griffith University DNA Sequencing Facility, Griffith University, Queensland for analysis on a ABI 3700 DNA sequencer.

CHAPTER 3

EPIDEMIOLOGY

3.1 Background

Examination of demographic data may provide important information into the spread of infection and those susceptible. As such, for all cases of culture proven campylobacteriosis, information such as the age and sex of the patient, area of residence and time of year of the infection are sent by Tasmanian pathology departments to the Tasmanian Public Health Morbidity Database (TPHMD) maintained by the Department of Public Health. Australia wide information is stored at the Communicable Diseases Network Australia – National Notifiable Disease Surveillance Database System (NNDSS) in Canberra, ACT.
Campylobacter isolates were collected from the Microbiology departments of the five major pathology providers around Tasmania; Royal Hobart Hospital, Launceston General Hospital, Hobart Pathology, Launceston Pathology and North West Pathology from January 1998 to March 1999. All strains of *Campylobacter* were originally isolated on Oxoid *Campylobacter* media with antibiotic supplement CAT SR174 added, and incubated at 42°C in an Oxoid 3.4 litre anaerobic jar with *Campylobacter* gas generating kits to generate a microaerophilic atmosphere. During this time period, 249 isolates were obtained from faecal samples of patients presenting with gastroenteritis and one isolate from blood cultures.

3.2 *Campylobacter* Notifications

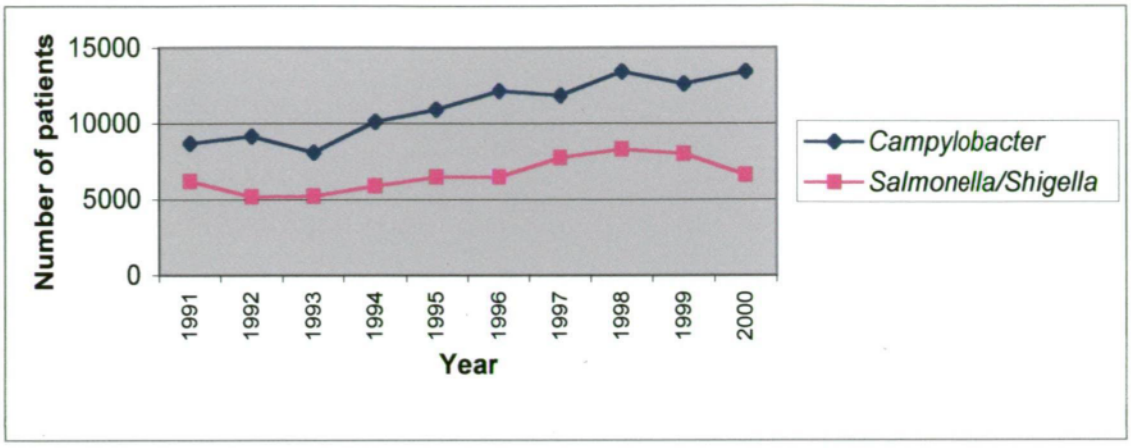
3.2.1 *Campylobacter* notifications in Australia

Data on *Campylobacter* infections in all states and territories of Australia (except NSW as campylobacteriosis was reported as 'foodborne disease' or 'gastroenteritis in an institution' and exact numbers a therefore not known) are held in the NNDSS and have shown a steady increase in numbers over the period 1991 – 2000 (Figure 3.1).
This increase may represent improvements in isolation techniques or increased reporting of isolations over the last 10 years. In comparison, notifications of *Salmonella* and *Shigella* combined do not exceed total *Campylobacter* isolations over the corresponding time period and have remained relatively stable.

3.2.2 *Campylobacter* notifications in Tasmania

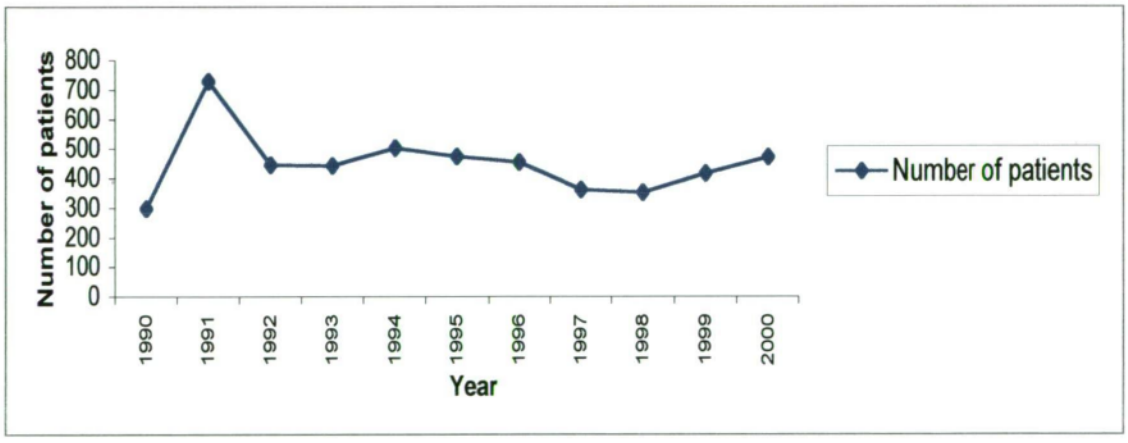
Numbers given to the database over the period of 1990 – 2000 show are relatively stable isolation rate in Tasmania except for a sharp peak in 1991 (Figure 3.2).

Figure 3.1: Number of *Campylobacter, Salmonella/Shigella* isolations in Australia for the years 1991–2000.



Communicable Diseases – Australia (NNDSS notification table) – Information does not include NSW.
http://www.health.gov.au/pubhlth/cdi/nndss/year005.htm

Figure 3.2: Tasmania *Campylobacter* notifications 1991–2000



Source: Year 1990:Tasmanian Public Hospital Morbidity Database
Years 1991-2000: Communicable Diseases – Australia (NNDSS notification table)
http://www.health.gov.au/pubhlth/cdi/nndss/year005.htm

3.2.3 Rates of *Campylobacter* infection

The rate of *Campylobacter* infection per 100,000 population in Tasmania was less than the Australia-wide average (99.2 and 112.6 respectively), mainly due to the higher rates of infection experienced in the Northern Territory and South Australia (Table 3.1). While equivalent case numbers were seen in both the south and north of Tasmania over the time period studied (January 1998 – March 1999), figures obtained from the TPHMD show that over the longer period 1990-97, the rate of infection per 100,000 population was higher in the north of the state compared to the south (118 and 94.7 respectively) (Figure 3.3). Reports to the Centers for Disease Control and Prevention in the USA have shown the annual infection rate seen in that country to be 5-6 per 100,000 population. However more intensive reporting from population based studies estimate the true rate to be closer to 1000 per 100,000 population with under-reporting and testing by general practitioners the likely cause of the discrepancy (Blaser, 1997).

Table 3.1: Total number of *Campylobacter* cases and rates per 100,000 population for Australian states, 1996

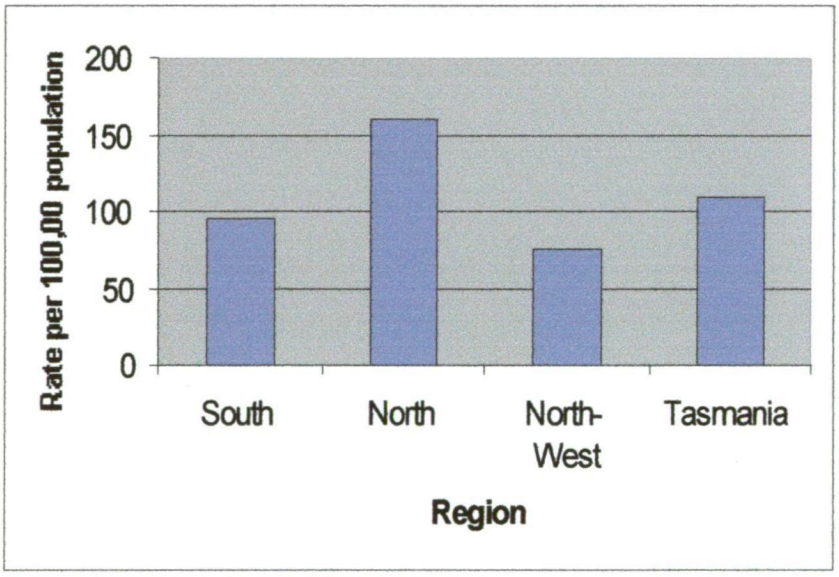
	ACT	NT	QLD	SA	TAS	VIC	WA	Total
No. of cases	**256**	**262**	**3030**	**2638**	**456**	**3555**	**1961**	**12158**
Rate/ 100,000	**85.5**	**134.3**	**89.9**	**184.7**	**99.2**	**81.3**	**113.6**	**AVG 112.6**

Compiled from: 1. Communicable Diseases – Australia (NNDSS notification table) – Information does not include NSW.
http://www.health.gov.au/pubhlth/cdi/nndss/year005.htm
2. Australian Bureau of Statistics: 1996 Census of Population and Housing
http://www.abs.gov.au/websitedbs

3.3 Guillain Barré syndrome notification rates in Tasmania

Up to two thirds of cases of GBS have a proven antecedent illness. Respiratory infections are the most commonly reported antecedent illness followed by gastrointestinal infections, of which *C. jejuni* is the most frequently identified cause. A documented outbreak of *Campylobacter jejuni* gastroenteritis in Jordan during 1978 and affecting >5,000 people, subsequently produced 16 cases of GBS, 8 to 24 days after the onset of diarrhoea (Khoury, 1978).

Figure 3.3: Age standardised notification rate for campylobacteriosis by region, Tasmania, 1990-97.



Source:Tasmanian Public Hospital Morbidity Database

The GBS rate for Tasmania over the period 1990-97 was only 2.3 per 100,000 population (TPHMD, Figure 3.4) compared with 106.4 per 100,000 population for *Campylobacter* infection rates over the same time period. Quite clearly *Campylobacter* infection does not always result in progression to GBS. Two possible explanations for this occurrence are that only some patients have a predisposition to developing this neurological disorder following campylobacterial infection or else an infrequently encountered strain of *Campylobacter,* possessing an unknown neurological pathogenic mechanism, leads to GBS.

3.4 Collection of Tasmanian *Campylobacter* isolates for this study

3.4.1 Annual Distribution

The isolation of *Campylobacter* species in Tasmania throughout the year as determined from isolates sent to the Royal Hobart Hospital, for this study is shown in Figure 3.5.
The peak of isolation rates occurred in August to October 1998 followed by a second peak in January 1999. This pattern of *Campylobacter* notifications was not seen throughout the rest of Australia, where one major peak occurred between October 1998 and January 1999 (Figure 3.6). Monthly data supplied to the Tasmanian Public Health Morbidity Database (TPHMD) maintained by the Department of Public Health for the period 1997-1999 also showed clearly the two major peaks occurring on a yearly basis (Figure 3.7).

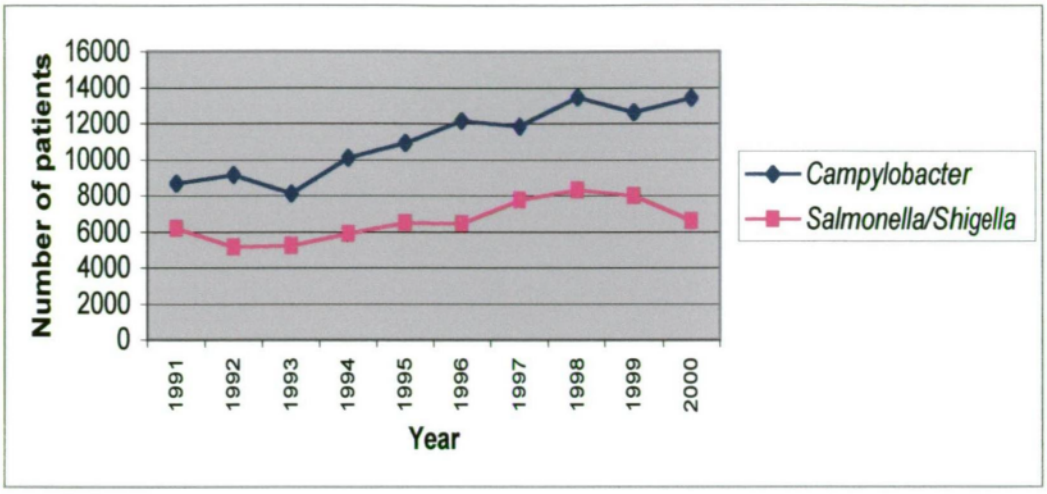
3.4.2 Age Distribution

Age distribution analysis of *Campylobacter* notifications shows a bimodal population (Figure 3.8) with major prevalence occurring in the less than 5 years of age and the 21 – 30 year age groups. This is in agreement with other studies of developed countries in which the highest age-specific attack rates occur in children < 1 year old with a second lower but broader peak in incidence occurring between 15 and 30 years of age (Blaser, 1997). In contrast, developing countries have only one major peak, occurring in children less than 2 years of age (Calva *et al*, 1988).

3.4.3 Sex Distribution

A slightly higher isolation rate of *Campylobacter* species was seen in male patients presenting with gastroenteritis, making up 55.8% of the patient's studied. Other epidemiological studies have also found slightly higher rates of *Campylobacter* infection in males (Blaser, 1997) and also higher rates of GBS with a male to female ratio of 1.25:1 (Hughes and Rees, 1997).

Figure 3.4: Age–standardised hospital separation rate for Guillain-Barré syndrome (principle diagnosis), Tasmania, 1990-1997.



Source:Tasmanian Public Hospital Morbidity Database

Figure 3.5: Number of isolates of *Campylobacter* species in Tasmania by month

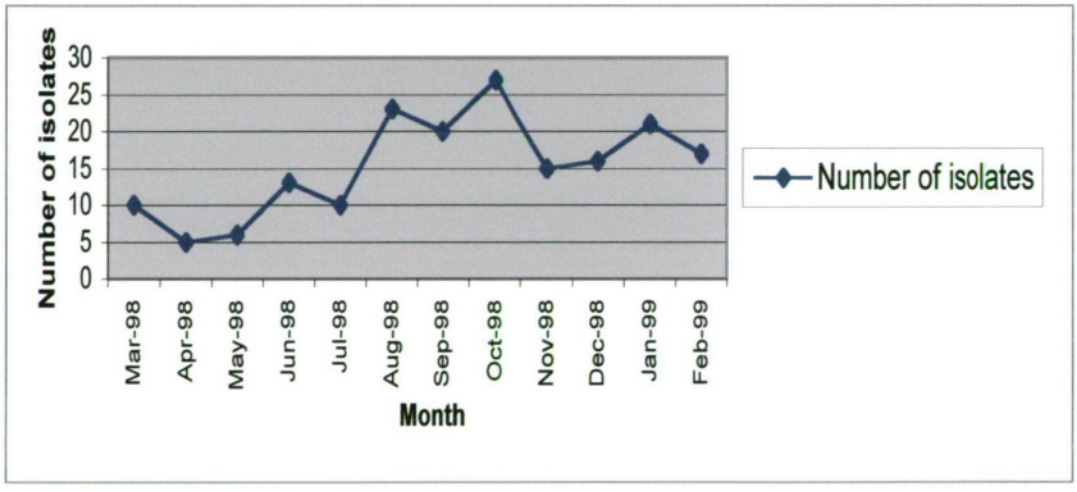
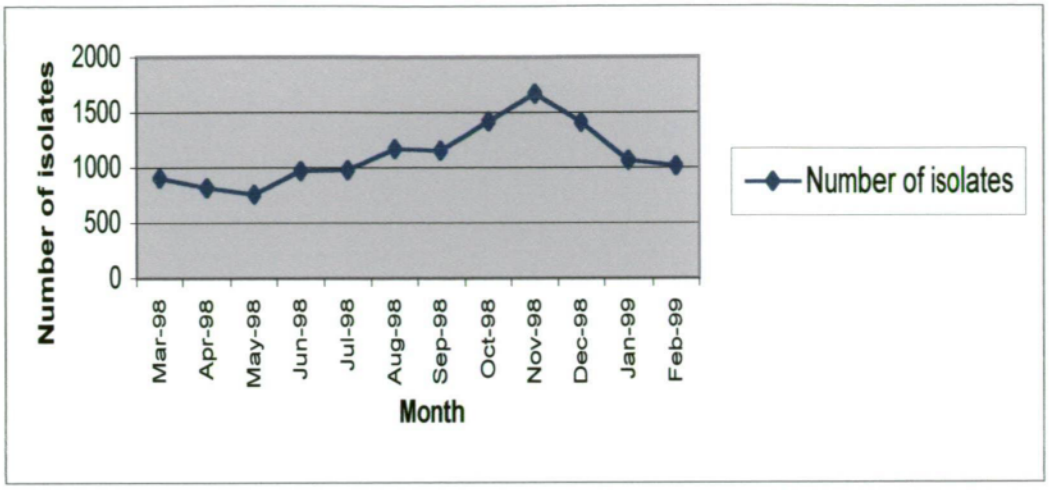
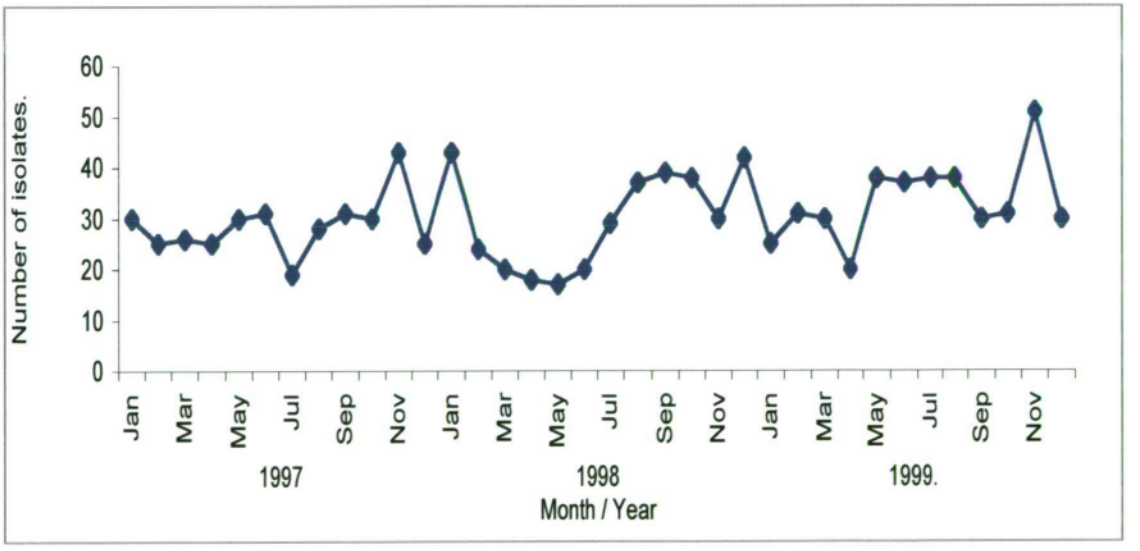


Figure 3.6: Number of isolates of *Campylobacter* species in Australia by month



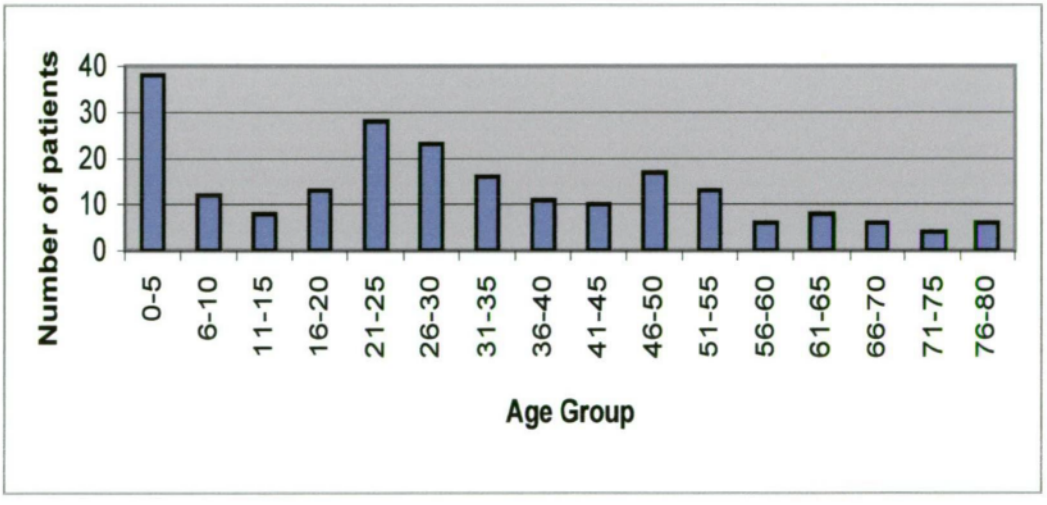
Communicable Diseases – Australia (NNDSS notification table) – Information does not include NSW.
http://www.health.gov.au/pubhlth/cdi/nndss/year005.htm

Figure 3.7: Number of isolates of *Campylobacter* species in Tasmania by month (1997-1999)



Source: Tasmanian Public Hospital Morbidity Database

Figure 3.8: Age distribution of patients presenting with gastroenteritis caused by *Campylobacter* species in Tasmania.



3.4.4 Intra-state laboratory compliance rates

All laboratories in the state were contacted prior to commencing this study and all agreed to pass on *Campylobacter* isolates for this study. Compliance rates of the laboratories to pass on their isolates to the study laboratory can be seen in Figure 3.9. Isolates from the south of the state were more frequently sent to the testing laboratory which is also located in the southern half of the state. This is in spite of the fact that an excellent daily transport system around the state between pathology providers was in place at the time of the study. Isolates from the two pathology providers in the south (Hobart Pathology and Royal Hobart Hospital) contributed 67% of their isolates (as determined by the notification rates from these laboratories) to the study compared with 35% for northern Tasmania laboratories (Launceston Pathology and Launceston General Hospital) and 25% from the north-west regional pathology laboratories (North-West Pathology).

3.5 Epidemiological typing of *Campylobacter* isolates using Pulsed Field Gel Electrophoresis (PFGE)

The ability of PFGE as an epidemiological typing tool has been well documented for a range of bacteria and in particular for suspected outbreaks of gastoenteritis caused by *C. jejuni* (Jackson *et al*, 1996) and *C. hyointestinalis* (Salama *et al*, 1992).

3.5.1 Method

PFGE was performed on all isolates as given in Section 2.9 Pulsed Field Gel Electrophoresis. As the aim of PFGE is to produce an electrophoresis gel pattern consisting of a few, high molecular weight DNA fragments, the choice of restriction endonuclease used depends upon the G+C ratio of the genomic DNA being cleaved. As *Campylobacter* species genomic DNA has a G+C ratio of ~ 35% (i.e. relatively low content), a restriction enzyme with a 6 base pair recognition sequence such as *Sma*I which recognises the DNA sequence "CCCGGG", was chosen. A RFLP software package from AAB (Advanced American Biotechnology) was chosen to detect and calculate molecular weights of the individual bands seen on electrophoresis gels. Patterns of isolates were also stored in the AAB software database for comparison of new isolates and dendrograms produced to show relative similarities between isolates.

3.5.2 Results

PFGE patterns obtained after *Sma*I digestion of genomic DNA contained between 4 to 11 bands varying in size from ~ 50 kbp to 600 kbp in size. Figure 3.10 demonstrates a representative PFGE gel of Tasmanian strains.

Figure 3.9: Isolation of *Campylobacter* species In Tasmania by region.

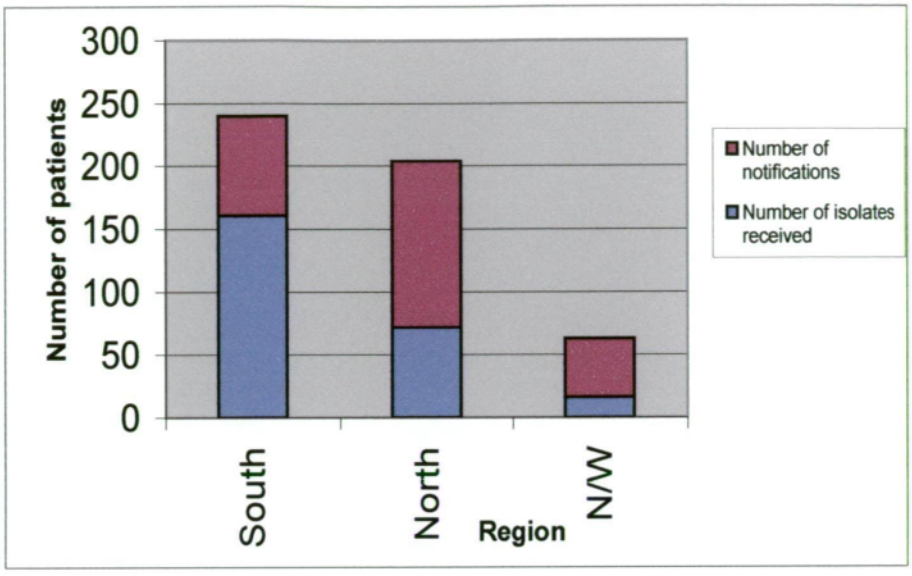
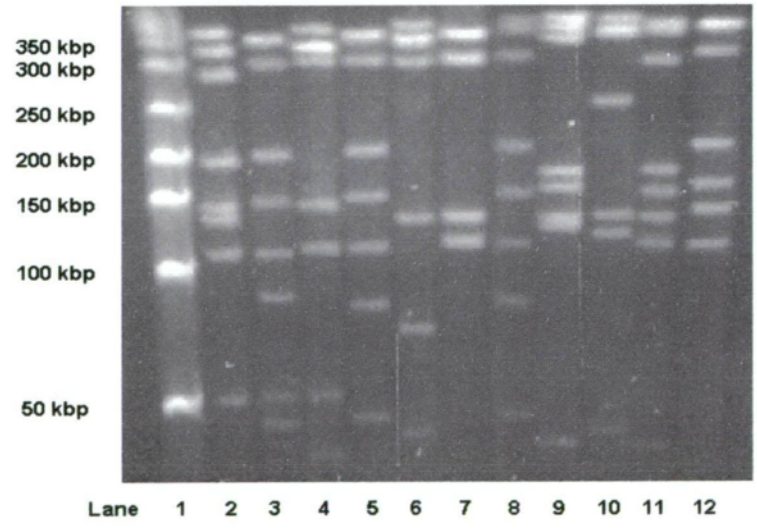


Figure 3.10: Pulsed Field Gel Electrophoresis of *Campylobacter jejuni* strains restricted with *Sma*I



1% agarose gel stained with ethidium bromide
Lane 1: Molecular Weight Marker, Lane 2-12 Tasmanian enteritis isolates of *C. jejuni*

Using a percentage similarity cutoff of 95%, computer analysis discriminated 77 different *Sma*I patterns (Table 3.2), with *C. jejuni* producing 68 different profiles, 7 *C. coli* profiles and 2 *C. lari* profiles. Of 250 isolates, 8 *Campylobacter* isolates did not produce a RFLP when *Sma*I was used as the cutting agent. These 8 isolates (7 *C. jejuni* and 1 *C. upsaliensis*) were therefore digested with *Sal*I, which recognises the genomic DNA sequence "GTCGAC" which resulted in 6 different RFLP profiles (Figure 3.11).

Table 3.2: Number of PFGE profiles following digestion with *Sma*I and *Sal*I

Number of Isolates	Identification	Different profiles: digested with *Sma*I	Different profiles: digested with *Sal*I
242	230 *C. jejuni*	68	NT
	10 *C. coli*	7	NT
	2 *C. lari*	2	NT
8	7 *C. jejuni*	No profile	5
	1 *C. upsaliensis*	No profile	1

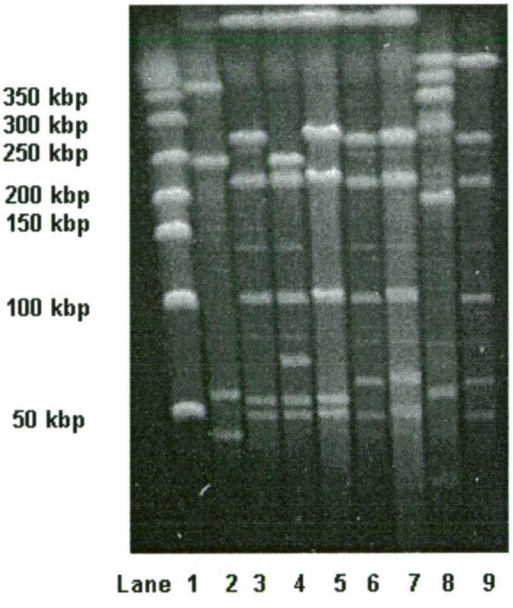
(NT: not tested)

By using the unweighted-pair group method with arithmetic averages (UPGMA) using simple band match, with a tolerance of 3.2%, dendrograms were produced to demonstrate the relationship between strains. Clustering can be seen within species and between *C. jejuni* and *C. coli* using *Sma*I (Figure 3.12). This affect can also be seen when *Sal*I was used differentiate *C. jejuni* from *C. upsaliensis* for the untypable isolates (Figure 3.13).

The ability of PFGE to differentiate between *C. jejuni* and *C. coli* strains has been previously reported (de Boer *et al*, 2000), as has its use to subspeciate both *C. fetus* and *C. hyointestinalis* (On, 2001). Large scale studies as to the usefulness of PFGE to distinguish the other *Campylobacter* species have not yet been investigated. A PFGE study of 21 strains of *C. fetus* clearly shows a homology between strains and a uniqueness that would separate them also from other *Campylobacter* species (Fujita *et al*, 1995).

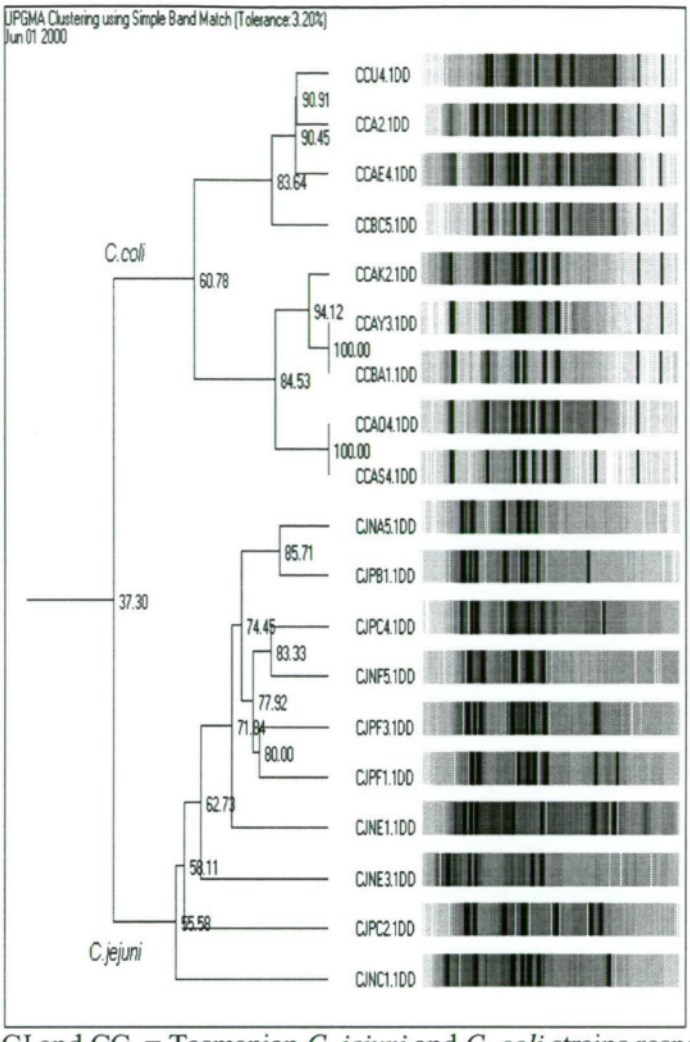
While the 250 *Campylobacter* strains studied could be separated into 83 different PFGE profiles, 28 % of strains were made up of only three patterns (Types 2, 11 & 28). A further 33% of strains belonged to seven more patterns (Types 1, 6, 10, 12, 15, 17 & 21) with the remaining 98 strains able to be separated into 73 types (Figure 3.14). This distribution of strains is typically found in other studies, with the majority of isolates belonging to a few genotypes only (Hanninen *et al*, 2000).

Figure 3.11: Pulsed Field Gel Electrophoresis of *Campylobacter* species strains restricted with *SalI*



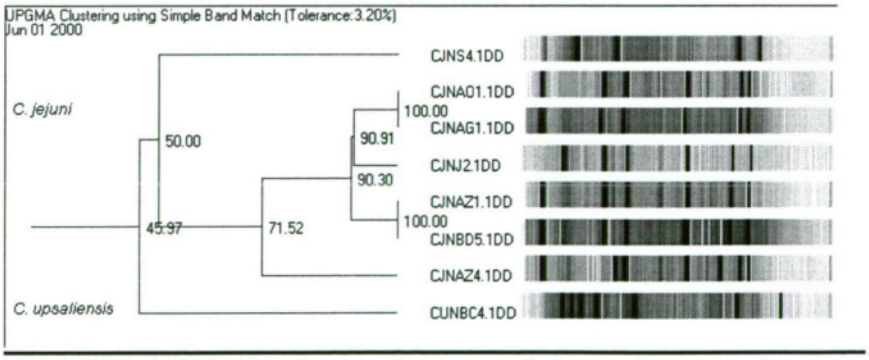
1% agarose gel stained with ethidium bromide
Lane 1: Molecular Weight Marker, Lane 2-7,9: *C. jejuni*, Lane 8: *C. upsaliensis*

Figure 3.12: PFGE dendrogram analysis of *C. jejuni* and *C. coli* isolates following restriction with *Sma*I



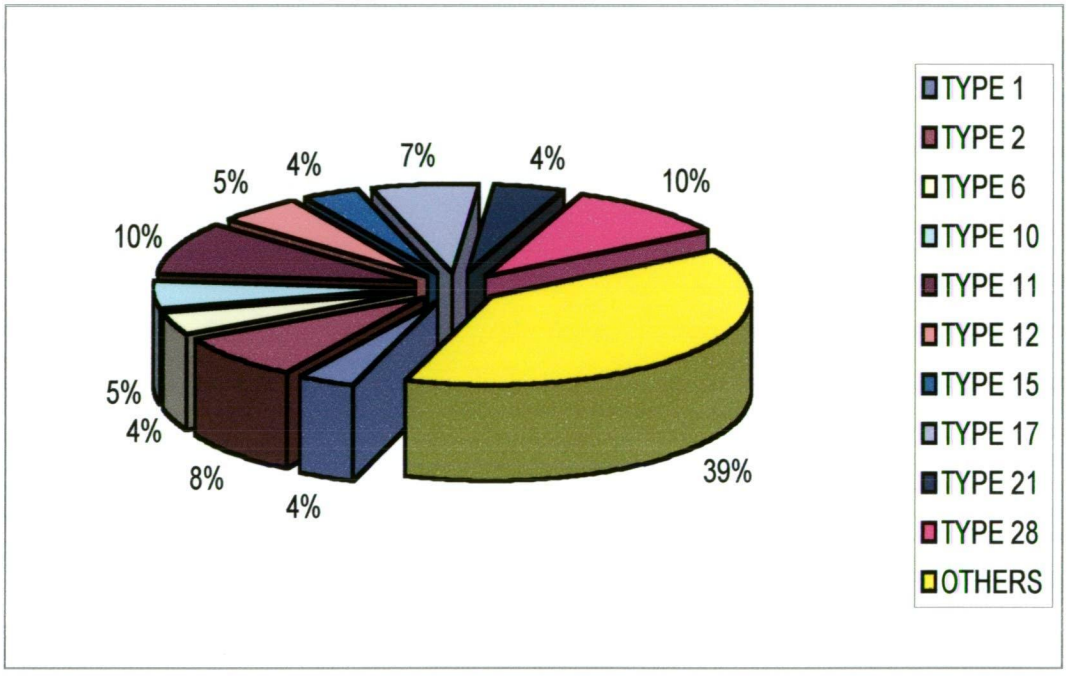
Strains prefexed CJ and CC = Tasmanian *C. jejuni* and *C. coli* strains respectively
Dendograms produced by the unweighted-pair group method with arithmetic averages (UPGMA) using simple band match, with a tolerance of 3.2%. (RFLP software: AAB)

Figure 3.13: PFGE dendrogram analysis of *Campylobacter* isolates untypable with *Sma*I and restricted with *Sal*I



Strains prefexed CJ and CU = Tasmanian *C. jejuni* and *C. upsaliensis* strains respectively
Dendograms produced by the unweighted-pair group method with arithmetic averages (UPGMA) using simple band match, with a tolerance of 3.2%. (RFLP software: AAB)

Figure 3.14: Distribution of Tasmanian *Campylobacter* PFGE types



3.5.3 Discussion

The usefulness of PFGE as an epidemiological tool can be demonstrated by plotting the isolation rates of particular genotypes over time. For example, one of the common genotypes, type 11, was seen throughout the entire time period studied in small numbers only. In the last month of the study, a marked increase in the isolation of type 11 was noted, comprising of 53% of all isolates typed for that month (Figure 3.15).

With this information, public health authorities can be notified about a possible outbreak of *Campylobacter*. Questioning those patients with this strain could elucidate the common source of infection.

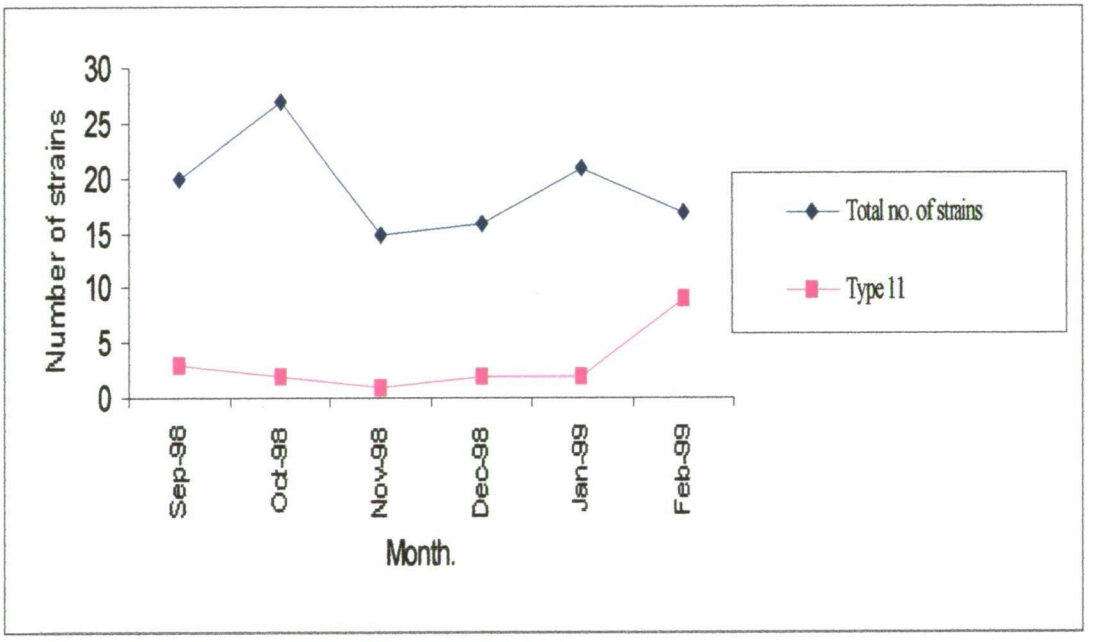
In addition, those patients with PFGE profiles seen infrequently but isolated within a short period of time would probably have the same source. During September of 1998, a seven year old male presented to the casualty department of Launceston General Hospital in the north of the state, complaining of diarrhoea. The *Campylobacter* strain isolated from this patient had not been encountered in the study to date but one week later, a strain with the same PFGE profile was isolated from a 20 year old male by Hobart Pathology in the south. The evidence suggests that these two cases are linked even though they were isolated more than 200 km apart. The same strain has not been isolated since.

3.6 Summary

Epidemiological information gained from examination of demographic data has provided useful information in the spread of *Campylobacter* within Tasmania. Whilst rates of infection per 100,000 population in Tasmania are less than the national average, the north of the state has a higher infection rate than the population in the south. Furthermore, increased isolation rates are seen in both early spring and summer in Tasmania in contrast to the national trend of only one peak in summer. Further investigation is therefore needed to determine the unusual distribution of *Campylobacter* infection within Tasmania and the seasonal irregularities that are seen. Increased knowledge of the causes will then allow intervention with a subsequent improvement in public health of the state.

Many new molecular based epidemiological methods are now available for the tracking of bacterial infections. One such method, PFGE, was used in this study to characterise the genotypes of *Campylobacter* present in the community between January 1998 and March 1999. Information gained from this study allows public health authorities to improve tracking of potential outbreaks of gastroenteritis caused by this bacterium by determining increased prevalence rates of particular strains in the community. Furthermore, subtyping of *Campylobacter* isolates by PFGE, may elucidate which particular genotypes are associated with diseases such as GBS and allow a more reliable prediction of outcome for those infected with these strains.

Figure 3.15: Number of PFGE type 11*C. jejuni* isolated in Tasmania by month



CHAPTER 4

DEVELOPMENT OF A *CAMPYLOBACTER* SPECIES IDENTIFICATION SCHEME

4.1 Aim

Over recent years, the genus *Campylobacter* has grown and now includes a number of non-*C. jejuni* species that are occasionally isolated from the faecal samples of patients presenting with gastroenteritis. A large scale PCR-based study by Lawson *et al* (1999), determined that *C. jejuni/C. coli* represented 97% of all enteropathogenic campylobacters causing diarrhea with *C. upsaliensis* 2.2%, *C. hyointestinalis* 0.6% and *C. lari* 0.2% making up the balance of the isolates. Most laboratories however only identify isolates to the genus level due to the difficulties involved in identifying this group of organisms. In the present study isolates were collected from around Tasmania in order to determine the prevalence of various species causing diarrhea within the Tasmanian community. Furthermore, while the majority of strains isolated from GBS patients have been *C. jejuni,* other species such *C. upsaliensis* have also been implicated (Ho *et al*, 1997). Accurate speciation of isolates may therefore provide further information about those strains that are neuropathogenic. The aim was to therefore determine a robust and rapid identification system based upon a multiplex PCR (16S rRNA/Hippurate gene)/Dot Blot Hybridisation test and to compare the accuracy of this test for the speciation of *Campylobacter* isolates to standard biochemical tests.

4.2 Background

4.2.1 Biochemical identification

Most Australian medical laboratories only identify *Campylobacter* isolates to the genus level as they lack the expertise and range of tests needed for speciation (RCPA: QAP results, 2002). When relying upon a limited number of tests, even the commonly isolated *C. jejuni* can be misidentified, particularly when hippurate negative strains are encountered (Morris *et al*, 1985). To overcome this problem, several researchers have produced identification schemes based upon a wide range of biochemical reactions. One of the earlier attempts was a dual-purpose identification and biotyping scheme developed by Bolton, Holt and Hutchinson (1984). Twelve biochemical tests were used to produce a numerical code which was then compared to a database of reference strain codes. The original scheme differentiated the six species known at that time therefore, application of this system today, following the discovery of a number of new species in subsequent years, renders this scheme obsolete. However, a number of reference laboratories

continue to use the Bolton scheme for the valuable information it provides as a biotyping and epidemiological tool.

More recently, computerised schemes based upon probability matrices have been used with more success. A commercial identification kit for campylobacters produced by Biomerieux Ltd. (API Campy) utilising this system can differentiate 18 species and subspecies with 21 tests. While this system provides an easy to use, standardised method that is available to all laboratories, no large-scale studies have been performed to evaluate accuracy of identification to the species level. Several reports have highlighted discrepancies while utilising this kit such as the misidentification of *C. concisus* as *C. mucosalis* (On, 1994) and *Arcobacter butzleri* as either *C. cinaedi* or *A. cryaerophilus* (Jacob, Lior and Feuerpfeil, 1993). While smaller studies have evaluated this kit, they have only assessed its ability to identify the more commonly isolated *C. jejuni*, *C. coli* and *C. lari* (Huysmans, Turnidge and Williams, 1995). Another identification matrix with 18 biochemical tests was recently evaluated and could differentiate between 37 species and subspecies of *Campylobacter, Arcobacter, Helicobacter* and related organisms (On, 1996b). While the study confirmed that this matrix was useful for identifying typical strains, difficulties were encountered when trying to differentiate between certain species.

4.2.2 Molecular identification

In recent years, the introduction of DNA-DNA hybridisation studies has been a major advancement in the identification of bacteria. Although providing definitive species identification, the method was time consuming requiring special facilities for the handling of radioactivity and was also not optimised for rapid identification of large numbers of isolates. A number of other molecular techniques have been developed over subsequent years to differentiate this group of bacteria. Firstly, nucleic acid probes that are genus or species specific have been described. These include genus specific probes designed from 16S rRNA gene sequence data for *Arcobacter* spp. (Wesley *et al*, 1995) and species specific probes for *C. jejuni* subsp. *jejuni, C. coli, C. lari, C. fetus* subsp. *fetus, C. hyointestinalis, C. sputorum* and *C. upsaliensis* (Chevrier *et al*, 1989). A number of commercial kits are also available based on 16S rRNA gene sequences and detect the more common thermophilic campylobacters, *C. jejuni* subsp. *jejuni, C. coli* and *C. lari*. These include the SNAP system (Syngene, San Diego, Calif.) and AccuProbe (Gen-Probe Inc., San Diego, Calif.) The first system requires immobilization of target DNA on a suitable membrane before hybridisation to alkaline-phosphatase labeled probes, while the AccuProbe is a chemiluminescence assay performed in solution and requiring a luminometer for reading of results.

The polymerase chain reaction (PCR) has revolutionised studies of bacterial identification. Many of these assays are designed using conserved and variable regions of the 16S rRNA gene sequence to differentiate bacteria to the genus and species level. PCR amplification of the 16S rRNA gene of *Campylobacter, Arcobacter* and *Helicobacter* species and subsequent restriction enzyme digestion has been used to identify to the genus (Marshall *et al*, 1999) and species levels (Harrington *et al*, 1999).

Multiplex PCR using both 16S rRNA and other *Campylobacter* genes has also been used to differentiate the two most commonly isolated human pathogens *C. jeuni* and *C. coli*. Isolates containing the 857 bp 16S rRNA *Campylobacter* specific amplicon as well as the 589 bp *map*A amplicon would be identified as *C. jejuni*. Similarly, if the isolate possessed the 857 bp genus specific fragment and the smaller 462 bp. *ceu*E amplicon, it would be designated *C. coli* (Denis *et al*, 1999). Other non-16S rRNA PCR tests include detection of the hippurate gene, *hip***O**, directly from faecal samples. This assay correctly identified both hippurate positive and hippurate negative isolates of *C. jejuni* from other *Campylobacter* species (Linton *et al*, 1997).

A PCR-enzyme linked immunosorbent assay (ELISA) was developed to identify *C. jejuni, C. coli, C. lari, C. upsaliensis, C. hyointestinalis, C. helveticus and C. fetus* directly from faecal samples (Metherell, Logan and Stanley, 1999). Initially, a PCR was performed from stool-extracted DNA or purified DNA from isolates to amplify a large 16S rRNA fragment and the product added to a microtitre tray bound with various internal 16S rRNA specific probes for each of these species. These results correlated with culture positive stools for *C. jejuni* and *C. coli*. In addition, 2 faecal samples produced PCR-ELISA results for the *C. hyointestinalis* and *C. helveticus* probes which were not subsequently cultured.

Therefore, not only is this dual detection and identification system a more rapid method than the current culture techniques, it also detects non-viable isolates of *Campylobacter* and possible multiple species infections.

4.3 Biochemical Identification

4.3.1 Methods

As the testing laboratory for this study was not a *Campylobacter* reference laboratory and had limited availability to various biochemical reagents, a basic set of tests was used in this study to identify the most commonly isolated *Campylobacter* species from humans, *C. jejuni*, with all other isolates being identified with a commercial multi-test system. The screening test set consisted of growth at 42°C, the hippurate test and susceptibilities to cephalothin and naladixic acid. All isolates which produced a positive hippurate test, grew at 42°C, and which were both

resistant to cephalothin and sensitive to naladixic acid were identified as *C. jejuni* subsp. *jejuni* as recommended by Nachamkin (1999). Those with the same biochemical pattern but which were sensitive to cephalothin were identified as the infrequently encountered *C. jejuni* subsp. *doylei.* While the commercially available biochemical identification system API Campy from bioMerieux was shown to be no more accurate than standard biochemical tests (Huysmans, Tunidge and Williams, 1995), it was chosen for non-*C. jejuni* isolate identification as it was readily available and easy to use.

4.3.2 Results

Campylobacter isolates were as given in Section 2.2: Sample collection and storage.
Of the 250 strains tested, 237 (94.8%) were identified as *C. jejuni* subsp. *jejuni (*235 by a positive hippurate hydrolysis and 2 with negative hippurate results subsequently identified by Campy API). The remaining 13 hippurate negative strains were identified as: 10 *C. coli* (4.0%), 1 *C. lari* (0.4%), 1 *C. upsaliensis* (0.4%) and 1 unidentified (0.4%) (Table 4.1).

Table 4.1: Biochemical identification of *Campylobacter* species

Number of isolates	Hippurate hydrolysis	Cephalothin Sensitivity	Naladixic Acid Sensitivity	Identification
233	POSITIVE	RESISTANT	SENSITIVE	*C. jejuni* subsp. *jejuni*
2	POSITIVE*	RESISTANT	RESISTANT	*C. jejuni* subsp. *jejuni*
15	NEGATIVE*	RESISTANT	SENSITIVE	2 *C. jejuni* subsp. *jejuni*
		RESISTANT	SENSITIVE	10 *C. coli*
		RESISTANT	RESISTANT	1 *C. lari*
		SENSITIVE	SENSITIVE	1 *C. upsaliensis*
		SENSITIVE	RESISTANT	1 Unidentified

* Identified by bioMerieux API Campy identification system

These results are similar to previous studies in the Netherlands in which *C. jejuni* (91.9%), *C. coli* (7.5%) and *C. upsaliensis* (0.6%) were also isolated utilising 5 different selective media and the filtration method (Endtz *et al*, 1991a). With such a wide variety of growth media supplemented by the filtration system, it is somewhat surprising that the Netherlands study did not isolate more

of the infrequently encountered species of *Campylobacter* and *Arcobacter*. Although the best culture schemes would include a variety of culture media at both 37°C and 42°C, large-scale studies have shown that the proportion of other *Campylobacter* and *Arcobacter* species causing diarrhea is relatively small. Whilst accepting that a few strains may only grow at 37°C and an occasional strain will be sensitive to the antimicrobial agents in the media, it would seem that Tasmanian diagnostic laboratories have not been greatly disadvantaged by utilising only one medium type at 42°C. Unfortunately, those few isolates that are missed may be associated with GBS and therefore accurate data on other *Campylobacter/Arcobacter* responsible for GBS may be lost.

The detection of two strains of *C. jejuni* subsp. *jejuni* with resistance to naladixic acid is not typical of this species. In one Australian study, Huysmans and Turnidge (1997) tested the susceptibilities of 100 human isolates of *Campylobacter* to various antimicrobial agents. These included 79 strains of *C. jejuni* subsp. *jejuni*, 19 *C. coli* and 2 strains of *C. lari*. Resistance to the quinolones was only found in *C. lari*, a marker commonly used for identification of this species. No *C. jejuni* or *C. coli* strains were found to be resistant to naladixic acid. Interestingly, they also found two strains of *C. jejuni* subsp. *jejuni* that were sensitive to cephalothin. Previous overseas studies in Finland and United Kingdom have reported increased resistance to naladixic acid and the fluoroquinolones in *C. jejuni* and *C. coli* (Rautelin, Renkonen and Kosunen, 1991; Bowler and Day, 1992). In these countries, widespread use of fluoroquinoles in animal husbandry, especially poultry farming has been implicated as a cause of increased resistance (Endtz *et al,* 1991b). As laboratories often rely on the resistance profiles of cephalothin and naladixic acid for identification, misidentification will occur as resistance to naladixic acid becomes more prevalent and atypical strains with variable sensitivities to cephalothin are encountered.

Two strains of hippurate negative *C. jejuni* subsp. *jejuni* were also found in the present study which were subsequently speciated by the API-Campy system. While relatively uncommon, hippurate negative *C. jejuni* have been reported in a number of studies (Nicholson and Patton, 1995; Totten *et al,* 1987). The basic hippurate hydrolysis tube test used by most researchers is based upon the detection of glycine within an incubated suspension of the bacteria. This is done by the addition of ninhydrin to the suspension and the development a purple colour within the tube. False negatives have been seen with a number of variations to this procedure with more sensitive results being reported with the use of gas-liquid chromatography (GLC) (Morris *et al*, 1985). However, subsequent reports showed the presence of some strains of *C. jejuni*, as confirmed by DNA-DNA hybridisation studies, to be hippurate hydrolysis negative even by GLC

(Totten *et al,* 1987). Clearly, the need for a definitive genetically based test to define speciation is needed.

4.4 Molecular Identification:

As the majority of *Campylobacter* species isolated from human faeces in patients suffering gastroenteritis are *C. jejuni* (CDI, 1997), a multiplex PCR was designed to both confirm that the isolates sent to our laboratory were indeed *Campylobacter* species or *Arcobacter* species and to quickly identify those that were *C. jejuni.* All non-*C. jejuni* isolates were further identified using the dot blot hybridisation with species-specific biotinylated probes to the 16S PCR product from the Multiplex PCR assay.

4.4.1 Multiplex PCR (16S sRNA/Hippurate gene)

4.4.1 (a) Method

Gene sequences for 16S rRNA and hippurate genes were obtained from Genbank using the nucleotide search engine at the National Centre for Biotechnology (NCBI) web site (http://www.ncbi.nlm.nih.gov). Designed primers were compared for specificity against Genbank's database using the computer program Basic BLAST (NCBI web site).
Crude bacterial DNA preparations were prepared by heating a MacFarland 1.0 suspension of bacteria (equivalent to a transmission of 70% as determined by a Vitek colorimeter), to 95°C for 10 minutes, after which the suspension was cooled to 4°C and used immediately for PCR. The optimised reaction mixture for PCR and the thermal cycling conditions using the Perkin Elmer DNA Thermal Cycler Model 9600 are given in Tables 4.2 and 4.3 respectively.

Table 4.2: Optimised reaction conditions for Multiplex PCR assay

Reagent	Final Concentration
10 X PCR Buffer II	1 X
Magnesium Chloride (mM)	4.2
Hippurate Sense Primer (µM)	0.45
Hippurate Anti-sense Primer (µM)	0.45
16S rRNA Sense Primer (µM)	0.45
16S rRNA Anti-sense Primer (µM)	0.90
dNTPs (dATP, dGTP, dCTP, dUTP)	200 µM of each
AmpliTaq® Gold DNA Polymerase	0.5 U
Water	Volume adjusted to 50 µL

Table 4.3: Optimised thermal cycling conditions for the Multiplex PCR

Amplification Step	Conditions
DNA Polymerase Enzyme activation (AmpliTaq® Gold DNA Polymerase)	5 minutes, 94°C
40 cycles – Denaturation	1 minute, 94°C
Annealing	1 minute & 20 seconds, 63°C
Extension	1 minute, 72°C
1 cycle – Final Extension	5 minute, 72°C

Based upon the 16S rRNA and hippurate genes (Figure 4.1), a 842 bp 16S rRNA amplicon fragment and 377 bp. hippurate amplicon was detected in isolates of *C. jejuni* with other species of *Campylobacter/Arcobacter* possessing only the 842 bp genus specific fragment. The identification scheme was complete after a 3 hour PCR cycle followed by 1hour detection on an agarose electrophoresis gel. Figure 4.2, demonstrates the presence of both PCR products in the *C. jejuni* control strain ATCC 700297 and ATCC 43431, and the larger 16S rRNA product in various other *Campylobacter* species and an *Arcobacter* species.

4.4.1 (b) Results

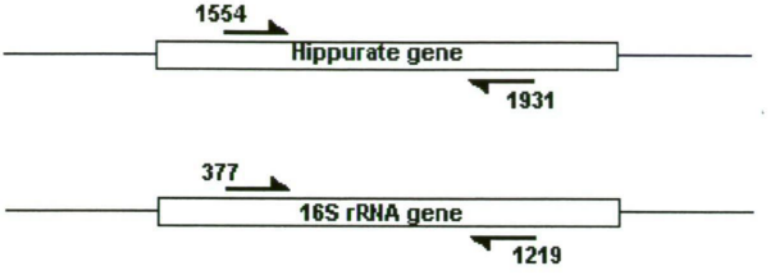
All isolates tested produced the larger 842 bp fragment with 237 strains also producing the smaller 377 bp fragment (Table 4.4). There were 13 isolates that only produced the larger 842 bp 16S rRNA product, signifying the presence of a non-*C. jejuni* species or *Arcobacter* species. The PCR product for these strains was used in the dot blot hybridisation test described below. The two *C. jejuni* control strains produced both PCR fragments with other *Campylobacter/Arcobacter* sp. control strains only producing the larger 842 b.p. fragment. Control strains that were not *Campylobacter* or *Arcobacter* did not produce DNA fragments of any size using the multiplex PCR.

4.4.2 Dot Blot Hybridisation

4.4.2 (a) Method

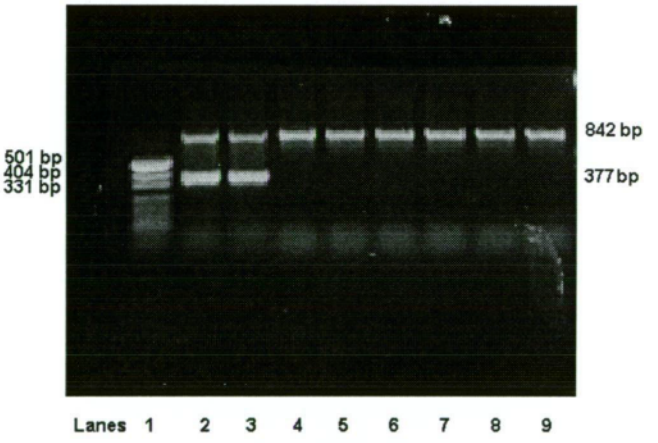
The Dot Blot Hybridisation assay was developed to speciate non *C. jejuni* isolates as determined by the multiplex PCR assay and was as given in Section 2.8 Dot Blot Hybridisation.
Biotinylated 16S rRNA probes were designed after identifying unique 16S rRNA sequences gene sequences (Figure 4.3) for *C. lari, C. upsaliensis, C. fetus* and *Arcobacter* species that were internal to the 16S rRNA gene fragment generated from the multiplex PCR (Table 4.5). The gene sequences were obtained from Genbank using the nucleotide search engine at NCBI. Probes used

Figure 4.1: PCR primers for the multiplex PCR (16S rRNA/Hippurate gene)



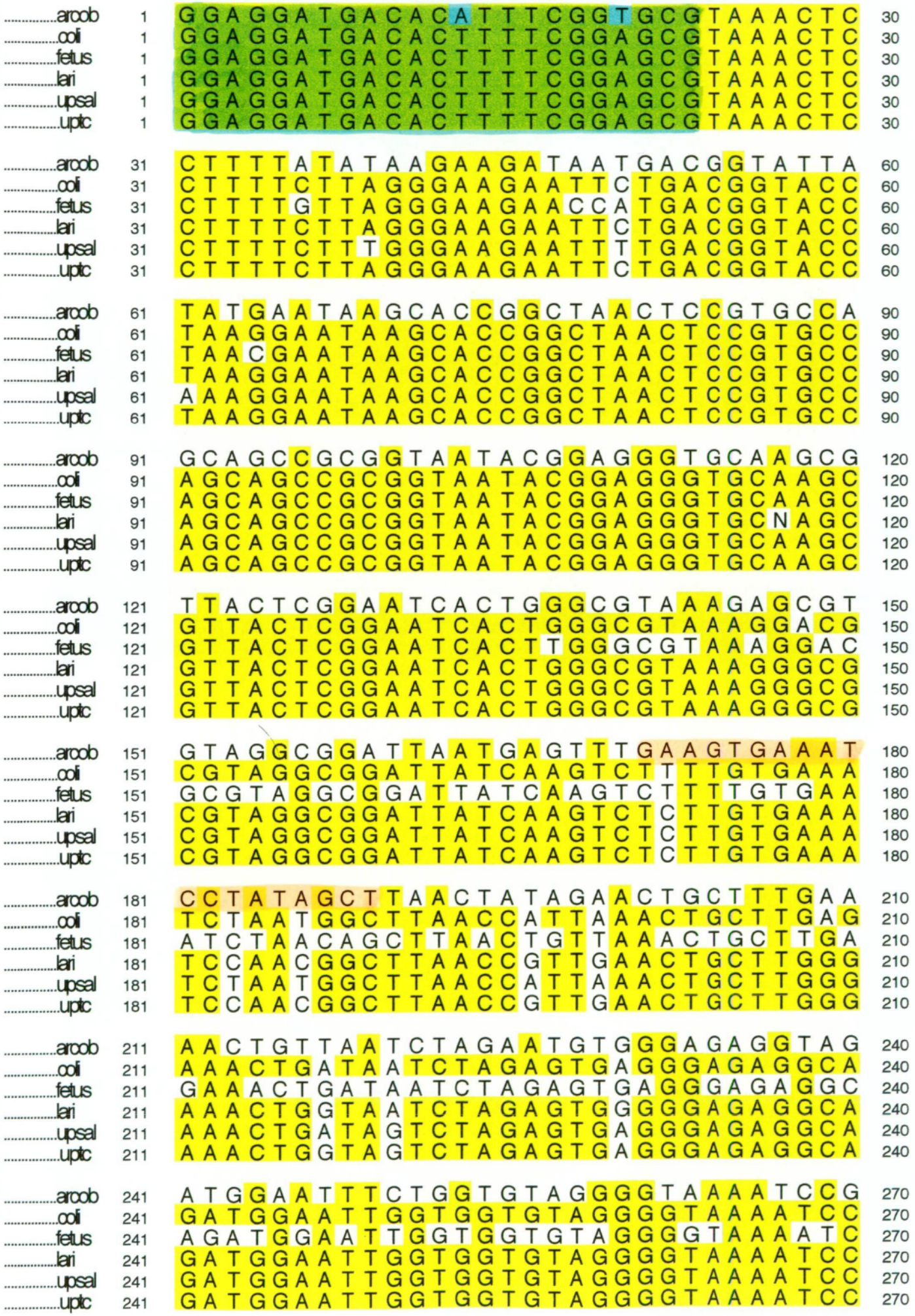
Source: Genebank – Hippurate gene (cjhippurc) , 16S rRNA gene: (*C. jejuni* strain 98/E599/10)

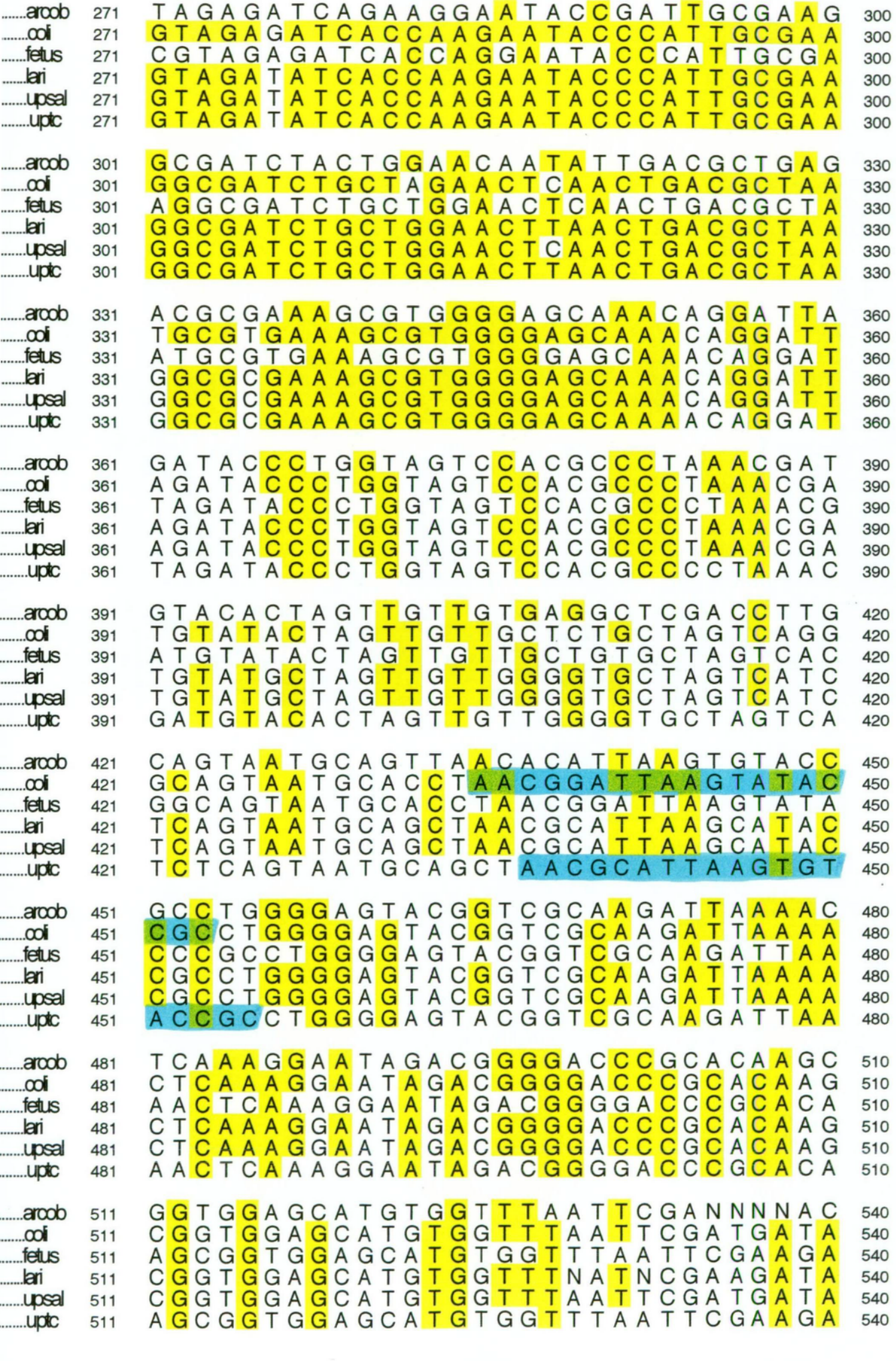
Figure 4.2: PCR products of various *Campylobacter* and *Arcobacter* strains using the Multiplex PCR for 16S rRNA and hippurate gene

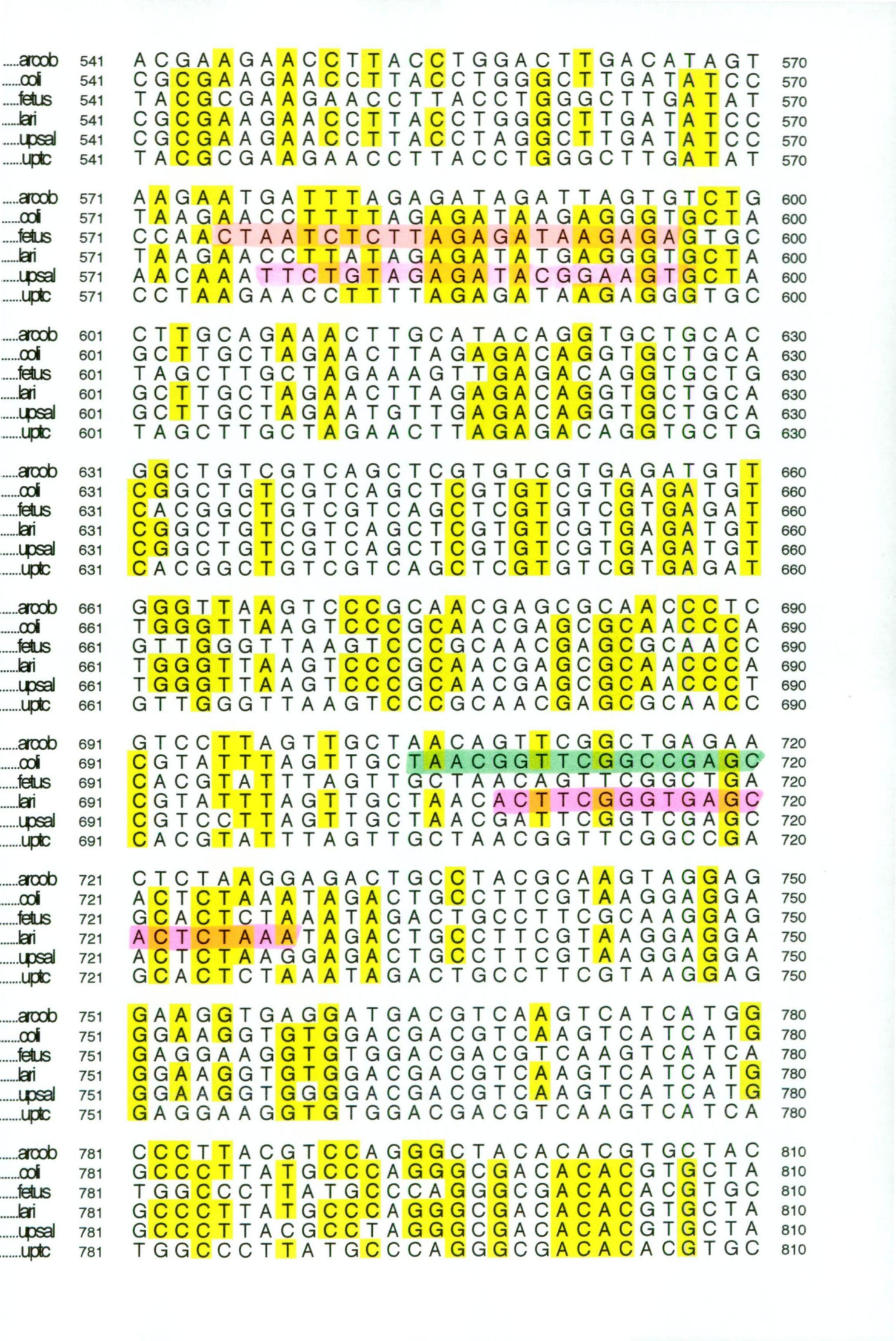


Lane 1: Molecular weight marker, 2: *C. jejuni* ATCC 700297, 3: *C. jejuni* ATCC 43431, 4: *C. coli* NCTC 11353, 5: *C. lari* ATCC 35221, 6: *C. fetus* NCTC 5850, 7: UPTC NCTC 11937, 8: *C. upsaliensis* Lab. Strain, 9: *Arcobacter* species Lab Strain.

Figure 4.3: Design of species specific probes: 16S rRNA sequence alignment for various *Campylobacter* and *Arcobacter* species




.....arcob 271 TAGAGATCAGAAGGAATACCGATTGCGAAG 300
.....coli 271 GTAGAGATCACCAAGAATACCCATTGCGAA 300
.....fetus 271 CGTAGAGATCACCAGGAATACCCATTGCGA 300
.....lari 271 GTAGATATCACCAAGAATACCCATTGCGAA 300
.....upsal 271 GTAGATATCACCAAGAATACCCATTGCGAA 300
.....uptc 271 GTAGATATCACCAAGAATACCCATTGCGAA 300
.....arcob 301 GCGATCTACTGGAACAATATTGACGCTGAG 330
.....coli 301 GGCGATCTGCTAGAACTCAACTGACGCTAA 330
.....fetus 301 AGGCGATCTGCTGGAACTCAACTGACGCTA 330
.....lari 301 GGCGATCTGCTGGAACTTAACTGACGCTAA 330
.....upsal 301 GGCGATCTGCTGGAACTCAACTGACGCTAA 330
.....uptc 301 GGCGATCTGCTGGAACTTAACTGACGCTAA 330
.....arcob 331 ACGCGAAAGCGTGGGGAGCAAACAGGATTA 360
.....coli 331 TGCGTGAAAGCGTGGGGAGCAAACAGGATT 360
.....fetus 331 ATGCGTGAAAGCGTGGGGAGCAAACAGGAT 360
.....lari 331 GGCGCGAAAAGCGTGGGGAGCAAACAGGATT 360
.....upsal 331 GGCGCGAAAAGCGTGGGGAGCAAACAGGATT 360
.....uptc 331 GGCGCGAAAAGCGTGGGGAGCAAAACAGGAT 360
.....arcob 361 GATACCCTGGTAGTCCACGCCCTAAACGAT 390
.....coli 361 AGATACCCTGGTAGTCCACGCCCTAAACGA 390
.....fetus 361 TAGATACCCTGGTAGTCCACGCCCTAAACG 390
.....lari 361 AGATACCCTGGTAGTCCACGCCCTAAACGA 390
.....upsal 361 AGATACCCTGGTAGTCCACGCCCTAAACGA 390
.....uptc 361 TAGATACCCTGGTAGTCCACGCCCCTAAAC 390
.....arcob 391 GTACACTAGTTGTTGTGAGGCTCGACCTTG 420
.....coli 391 TGTATACTAGTTGTTGCTCTGCTAGTCAGG 420
.....fetus 391 ATGTATACTAGTTGTTGCTGTGCTAGTCAC 420
.....lari 391 TGTATGCTAGTTGTTGGGGTGCTAGTCATC 420
.....upsal 391 TGTATGCTAGTTGTTGGGGTGCTAGTCATC 420
.....uptc 391 GATGTACACTAGTTGTTGGGGTGCTAGTCA 420
.....arcob 421 CAGTAATGCAGTTAACACATTAAGTGTACC 450
.....coli 421 GCAGTAATGCACCTAACGGATTAAGTATAC 450
.....fetus 421 GGCAGTAATGCACCTAACGGATTAAGTATA 450
.....lari 421 TCAGTAATGCAGCTAACGCATTAAGCATAC 450
.....upsal 421 TCAGTAATGCAGCTAACGCATTAAGCATAC 450
.....uptc 421 TCTCAGTAATGCAGCTAACGCATTAAGTGT 450
.....arcob 451 GCCTGGGGAGTACGGTCGCAAGATTAAAAC 480
.....coli 451 CGCCTGGGGAGTACGGTCGCAAGATTAAAA 480
.....fetus 451 CCCGCCTGGGGAGTACGGTCGCAAGATTAA 480
.....lari 451 CGCCTGGGGAGTACGGTCGCAAGATTAAAA 480
.....upsal 451 CGCCTGGGGAGTACGGTCGCAAGATTAAAA 480
.....uptc 451 ACCGCCTGGGGAGTACGGTCGCAAGATTAA 480
.....arcob 481 TCAAAGGAATAGACGGGGACCCGCACAAGC 510
.....coli 481 CTCAAAGGAATAGACGGGGACCCGCACAAG 510
.....fetus 481 AACTCAAAGGAATAGACGGGGACCCGCACA 510
.....lari 481 CTCAAAGGAATAGACGGGGACCCGCACAAG 510
.....upsal 481 CTCAAAGGAATAGACGGGGACCCGCACAAG 510
.....uptc 481 AACTCAAAGGAATAGACGGGGACCCGCACA 510
.....arcob 511 GGTGGAGCATGTGGTTTAATTCGANNNNAC 540
.....coli 511 CGGTGGAGCATGTGGTTTAATTCGATGATA 540
.....fetus 511 AGCGGTGGAGCATGTGGTTTAATTCGAAGA 540
.....lari 511 CGGTGGAGCATGTGGTTTNATNCGAAGATA 540
.....upsal 511 CGGTGGAGCATGTGGTTTAATTCGATGATA 540
.....uptc 511 AGCGGTGGAGCATGTGGTTTAATTCGAAGA 540


.....arcob 541 ACGAAGAACCTTACCTGGACTTGACATAGT 570
.....coli 541 CGCGAAGAACCTTACCTGGGCTTGATATCC 570
.....fetus 541 TACGCGAAGAACCTTACCTGGGCTTGATAT 570
.....lari 541 CGCGAAGAACCTTACCTGGGCTTGATATCC 570
.....upsal 541 CGCGAAGAACCTTACCTAGGCTTGATATCC 570
.....uptc 541 TACGCGAAGAACCTTACCTGGGCTTGATAT 570
.....arcob 571 AAGAATGATTTAGAGATAGATTAGTGTCTG 600
.....coli 571 TAAGAACCTTTTAGGAGATAAGAGGGTGCTA 600
.....fetus 571 CCAACTAATCTCTTAGAGGATAAGAGAGTGC 600
.....lari 571 TAAGAACCTTATAGAGATATGAGGGTGCTA 600
.....upsal 571 AACAAATTCTGTAGAGATACGGAAGTGCTA 600
.....uptc 571 CCTAAGAACCTTTTAGAGATAAGAGGGTGC 600
.....arcob 601 CTTGCAGAAACTTGCATACAGGTGCTGCAC 630
.....coli 601 GCTTGCTAGAACTTAGGAGACAGGTGCTGCA 630
.....fetus 601 TAGCTTGCTAGAAAGTTGAGACAGGTGCTG 630
.....lari 601 GCTTGCTAGAACTTAGGAGACAGGTGCTGCA 630
.....upsal 601 GCTTGCTAGAATGTTGAGGACAGGTGCTGCA 630
.....uptc 601 TAGCTTGCTAGAACTTAGGAGACAGGTGCTG 630
.....arcob 631 GGCTGTCGTCAGCTCGTGTCGTGAGATGTT 660
.....coli 631 CGGCTGTCGTCAGCTCGTGTCGTGAGGATGT 660
.....fetus 631 CACGGCTGTCGTCAGCTCGTGTCGTGAGAT 660
.....lari 631 CGGCTGTCGTCAGCTCGTGTCGTGAGGATGT 660
.....upsal 631 CGGCTGTCGTCAGCTCGTGTCGTGAGGATGT 660
.....uptc 631 CACGGCTGTCGTCAGCTCGTGTCGTGAGAT 660
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.....coli 661 TGGGTTAAGTCCCGCAACGAGCGCAACCCA 690
.....fetus 661 GTTGGGTTAAGTCCCGCAACGAGCGCAACC 690
.....lari 661 TGGGTTAAGTCCCGCAACGAGCGCAACCCA 690
.....upsal 661 TGGGTTAAGTCCCGCAACGAGCGCAACCCT 690
.....uptc 661 GTTGGGTTAAGTCCCGCAACGAGCGCAACC 690
.....arcob 691 GTCCTTAGTTGCTAACAGTTCGGCTGAGAA 720
.....coli 691 CGTATTTAGTTGCTAACGGTTCGGCCGAGC 720
.....fetus 691 CACGTATTTAGTTGCTAACAGTTCGGCTGA 720
.....lari 691 CGTATTTAGTTGCTAACACTTCGGGTGAGC 720
.....upsal 691 CGTCCTTAGTTGCTAACGATTCGGTCGAGC 720
.....uptc 691 CACGTATTTAGTTGCTAACGGTTCGGCCGA 720
.....arcob 721 CTCTAAGGAGACTGCCTACGCAAGTAGGAG 750
.....coli 721 ACTCTAAATAGACTGCCTTCGTAAGGAGGA 750
.....fetus 721 GCACTCTAAATAGACTGCCTTCGCAAGGAG 750
.....lari 721 ACTCTAAATAGACTGCCTTCGTAAGGAGGA 750
.....upsal 721 ACTCTAAGGAGACTGCCTTCGTAAGGAGGA 750
.....uptc 721 GCACTCTAAATAGACTGCCTTCGTAAGGAG 750
.....arcob 751 GAAGGTGAGGATGACGTCAAGTCATCATGG 780
.....coli 751 GGAAGGTGTGGACGACGTCAAGTCATCATG 780
.....fetus 751 GAGGAAGGTGTGGACGACGTCAAGTCATCA 780
.....lari 751 GGAAAGGTGTGGACGACGTCAAGTCATCATG 780
.....upsal 751 GGAAAGGTGGGGACGACGTCAAGTCATCATG 780
.....uptc 751 GAGGAAGGTGTGGACGACGTCAAGTCATCA 780
.....arcob 781 CCCTTACGTCCAGGGCTACACACGTGCTAC 810
.....coli 781 GCCCTTATGCCCAGGGCGACACACGTGCTA 810
.....fetus 781 TGGCCCTTATGCCCAGGGCGACACACGTGC 810
.....lari 781 GCCCTTATGCCCAGGGCGACACACGTGCTA 810
.....upsal 781 GCCCTTACGCCTAGGGCGACACACGTGCTA 810
.....uptc 781 TGGCCCTTATGCCCAGGGCGACACACGTGC 810

Biotinylated probe	
cc1	
cc2	
lari	
upsal	
fetus	
arco	

16S rRNA Multiplex PCR primers	
Forward	
Reverse	

Table 4.4: First stage identification using the Multiplex PCR for 16S rRNA and hippurate gene

Number of isolates	16S PCR positive	Hippurate PCR positive	Identification
250	237	237	*C. jejuni*
	13	0	non-*C. jejuni Campylobacter* or *Arcobacter* spp.
17 control strains	2	2	*C. jejuni* ATCC 700297 *C. jejuni* ATCC 43431
	6	0	*C. coli* NCTC 11353 UPTC NCTC 11937 *C. lari* ATCC 35221 *C. fetus* NCTC 5850 *C. upsaliensis* (Lab. Strain) *Arcobacter sp.* (Lab. Strain)
	0	0	*Proteus vulgaris* ATCC 8427 *Aeromonas hydrophila* ATCC 2433 *Pseudomonas aeruginosa* NCTC 10662 *Legionella pneumophila* ATCC 33152 *Escherichia coli* NCTC 11560 *Haemophilus influenzae* NCTC 4560 *Salmonella typhimurium* ATCC 3598 *Shigella flexneri* ATCC 12022 *Pseudomonas fluorescens* ATCC 13525

Table 4.5: Biotinylated 16S rRNA oligonucleotide probes to various *Campylobacter* and *Arcobacter* species

Probe	Organism Detected	Length	Sequence (5' to 3')	Reference
cc1	*C. coli* UPTC	22 n.t.	BiotinTTTGCGGTACACTT AA TGCGTT	Metherell, Logan and Stanley, 1999
cc2	*C. coli*	20 n.t.	BiotinTAAGCTCGGCCGAA CCGTTA	Metherell, Logan and Stanley, 1999
lari	*C. lari*	23 n.t.	BiotinATATTAGAGTGCTC ACCCGAAGT	this study
upsal	*C. upsaliensis*	23 n.t.	BiotinTAAACTTCCGTATC TCTACAGAA	this study
fetus	*C. fetus*	25 n.t.	BiotinATATCTCTTATCTCT AAGAGATTAG	this study
arco	*Arcobacter* species	22 n.t.	BiotinAATAGCTATAGGAT TTCACTTC	this study

UPTC: urea positive thermophilic *Campylobacter*; n.t.: nucleotides

for *C. coli* and urea positive thermophilic *Campylobacter* strains (UPTC) were from a previous study (Metherell, Logan and Stanley, 1999).

The hybridisation probe strategy is shown in Figure 4.4.

Incubation was performed overnight with biotinylated probes (concentration of 10 nM) cc1, cc2 and lari at 50°C to detect *C. coli* (Figure 4.5). UPTC and *C. lari*. *C. coli* strains are positive with both cc1 and cc2 probes; with the urea positive variant of *C. lari* (UPTC) positive in only the cc1 probe and *C. lari* positive with only the lari probe. If the isolates are negative using these probes, the hybridisation experiment is repeated at 43°C with the *C. upsaliensis* probe (Upsal) and then 39°C for *Arcobacter* spp probe (Arco) and then 34°C with the *C. fetus* probe (Fetus). The conjugate (Streptavidin-alkaline phosphate) was used in a 5% blocking solution (1:5000 dilution). Identification of control strains was confirmed by 16S rRNA sequencing as given in Section 2.12: DNA sequencing. As large epidemiological studies have shown that *C. jejuni* and *C. coli* are the most commonly isolated species (91.9% and 7.5% respectively)(Endtz *et al*, 1991a), using the multiplex PCR would identify the majority of strains as *C. jejuni* within 5 hours, with *C. coli* strains identified 24 hours later.

4.4.2 (b) Results/Discussion

Of the 13 isolates identified as non-*C. jejuni* campylobacters using the multiplex PCR, 10 were identified as *C. coli* using the cc1 and cc2 probes and 2 isolates bound the lari probe (*C. lari*). Dot blot hybridisation was performed at the lower temperature of 43°C using the *C. upsaliensis* probe which produced a positive identification in the remaining 1 unidentified strain. (Table 4.6)

The molecular based identification scheme identified all the *C. jejuni* subsp. *jejuni* strains found by API-Campy biochemical system including the two hippurate negative strains encountered. Also the 10 *C. coli*, 2 *C. lari* and 1 *C. upsaliensis* strains identified biochemically were correctly identified by the dot blot hybridisation assay. The single strain that could not be biochemically identified was found to be *C. lari* using the lari probe in the hybridisation assay. This isolate more closely resembled *C. fetus* being sensitive to cephalothin and resistant to naladixic acid. It also grew well at 37°C and poorly at 42°C, growth characteristics seen with isolates of *C. fetus*. Furthermore, *C. lari* sensitivity to cephalothin has not been reported previously, making this isolate quite atypical in its presentation.

Repeat hybridisation testing showed strong binding to the lari probe and no binding of the fetus probe. Unusual isolates have previously been reported following identification with

Figure 4.4: Flowchart for PCR/Dot Blot Hybridisation Assay

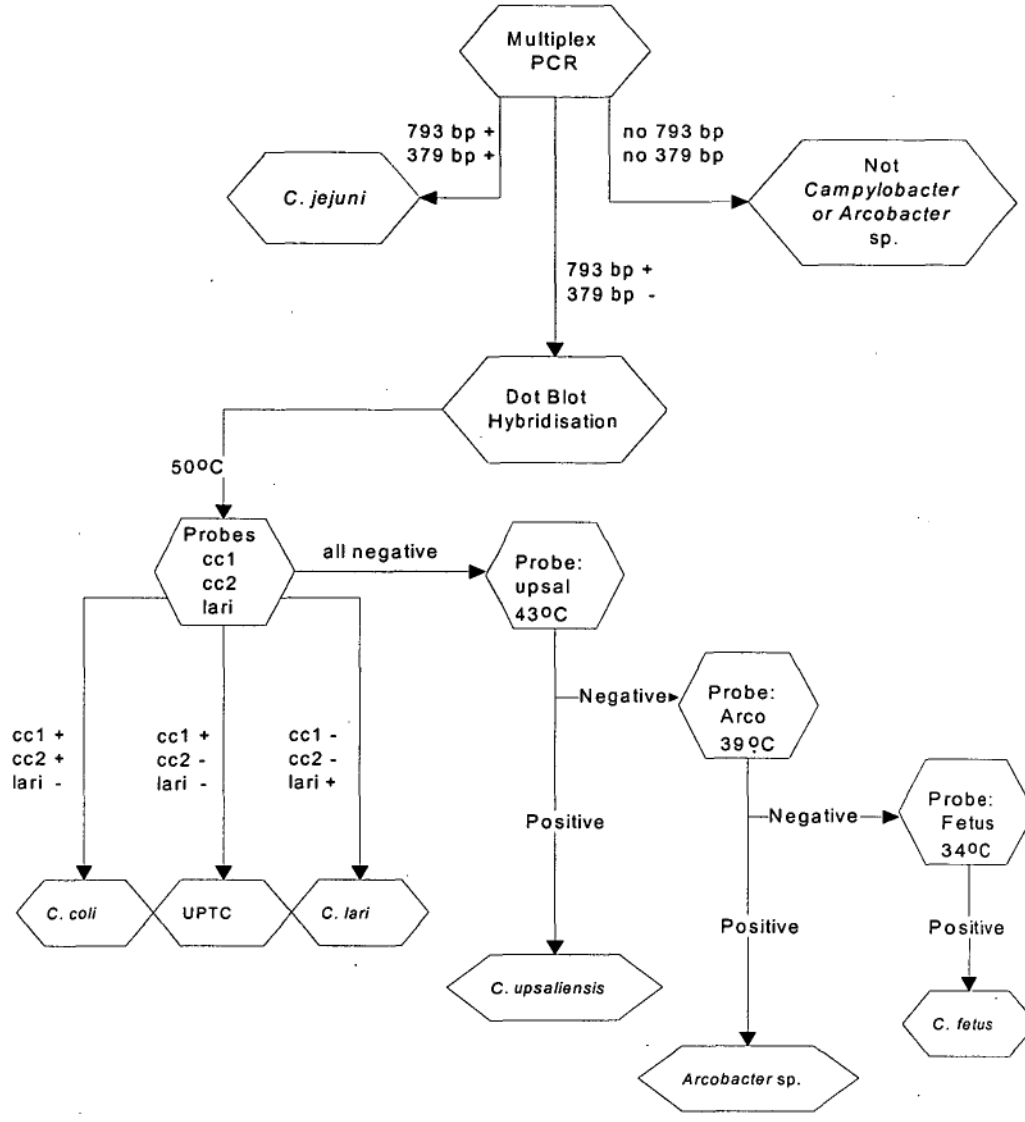
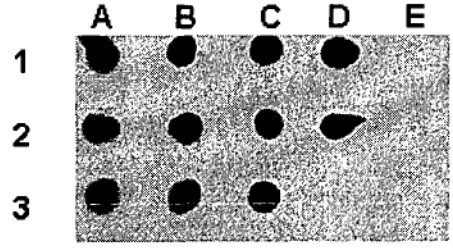


Figure 4.5: Dot blot hybridisation using the *C. coli* specific probe cc2



A1, A2, A3, B1, B2, B3, C1, C2, C3, D1: patient strains, D2: *C. coli* NCTC 11353, D3: *C. fetus* NCTC 5850, E1: *C. lari* patient strain 1, E2: *C. lari* patient strain 2, E3: *C. upsaliensis* patient strain

Table 4.6: Second stage identification using the dot blot hybridisation with probes internal to the 16S rRNA PCR product

Number of isolates	jej/col 1 probe positive	jej/col 2 probe positive	lari probe positive	upsal probe positive	Identification
13	10	10	0	NT	*C. coli*
	0	0	2	NT	*C. lari*
	0	0	0	1	*C. upsaliensis*
6 control strains	1	1	0	0	*C. coli NCTC 11353*
	1	0	0	0	*Urea positive thermophilic Campylobacter* (UPTC) *NCTC 11937*
	0	0	1	0	*C. lari ATCC 35221*
	0	0	0	0	*C. fetus NCTC 5850*
	0	0	0	1	*C. upsaliensis* Lab strain
	0	0	0	0	*Arcobacter* species Lab. strain

NT: not tested

the API-Campy identification system. Huysmans, Turnidge and Williams (1995), found that 3 out of 100 *Campylobacter* isolates were not identified by this system. DNA hybridisation studies subsequently identified those 3 isolates as being *C. coli* . They also concluded that conventional biochemical testing alone was satisfactory for the routine identification of thermophilic campylobacters. While our results agree with their findings, it should be noted that conventional biochemical testing alone would have failed to detect the hippurate negative strains of *C. jejuni* subsp. *jejuni* and to correctly identify unusually presenting strains such as the *C. lari* isolate encountered in our study.

4.5 Summary

The simple biochemistry scheme correctly identified the majority of *C. jejuni* subsp. *jejuni* isolates encountered in this study and is still useful to rapidly identify the species that is most

commonly isolated by routine laboratories. While large numbers of non-*C. jejuni* isolates were not tested in this study, the bioMerieux API-Campy identification system identified 12/13 non-*C. jejuni* strains encountered and also correctly identified both hippurate negative *C. jejuni* subsp. *jejuni* strains and therefore should still be considered for smaller laboratories that have limited resources and the need for off-the-shelf identification systems. For larger reference laboratories, a molecular based identification scheme such as the multiplex PCR/dot blot hybridisation system would give a definitive result for those less frequently encountered species as well as detection of the hippurate gene for accurate identification of hippurate negative strains of *C. jejuni*.

An adaptation of the system is possible allowing for the development of a PCR-enzyme linked immunosorbent assay (ELISA) for detection of *Campylobacter* and *Arcobacter* species directly from faecal samples similar to the system developed by Metherell, Logan and Stanley (1999). Such a system would decrease identification time by eliminating the need for culturing the organism initially. Other advantages of such a system would be the detection of mixed species infections and also the identification of strains unable to grow on the media and incubation temperatures used in routine laboratories.

CHAPTER 5

POTENTIAL MARKERS OF *CAMPYLOBACTER* ASSOCIATED WITH THE DEVELOPMENT OF GBS

Studies of GBS associated strains of *Campylobacter* show that they, as a group have a number of attributes in common. Firstly, certain serotypes such as Penner serotype O:19, are isolated from GBS patients at an increased frequency compared to other serotypes. Secondly, GBS associated strains have cell wall structures that mimic human gangliosides such as Gm1. The prevalence of potentially neuropathogenic strains of *Campylobacter* may therefore be determined by testing for these attributes in Tasmanian gastrointestinal isolates.

5.1 Penner Serotype O:19 strains

5.1.1 Background

The epidemiology of *Campylobacter*-associated GBS has been made possible by the use of a number of different typing systems. One of the first schemes to produce valuable information was the Penner O serotyping system which could differentiate 60 types of *C. jejuni* and *C. coli*.

In one Japanese study using this technique, it was found that of 12 isolates of *C. jejuni* associated with GBS, 10 (83%) belonged to serotype O:19 (Kuroki *et.al*, 1993). In that same study, only 1.7% of 1,150 enteritis-associated isolates of *C. jejuni* belonged to serotype O:19. While serotype O:19 is over-represented in GBS patients, it would seem that the high isolation rate of this serotype in the Japanese study might be a regional population phenomenon. Geographical distribution of a particular serotype can also be seen in a study from South Africa. In that particular region, serotype O:41 was found in 6 of 9 (67%) isolates of *C. jejuni* from patients that developed GBS, compared with only 0.1% of enteritis isolates from patients in the same region. These are significant results considering that serotype O:19 was found to be three times more prevalent in this community than serotype O:41 (Wassenaar *et.al*, 2000). Other studies from around the world have reported serotypes O:19 and to a lesser degree O:41along with serotypes O:1, O:2, O:4, O:4 complex, O:5, O:10, O:16, O:23, O:37, O:44, O:64 (Nachamkin and Blaser, 2000).

As serotyping is expensive and restricted to reference laboratories and does not detect all GBS-associated strains (Endtz *et al*, 2000), alternate methods to detect those strains of *Campylobacter* would aid in the elucidation of the mechanism of GBS induction.

In 1998, a PCR for the detection of *C. jejuni* serotype O:19 strains was first published (Misawa , Allos and Blaser, 1998). The test detected a single base pair difference in the DNA gyrase subunit B gene of *C. jejuni* isolates. A total of 42 isolates (18 O:19 and 24 non-O:19

strains) from five different countries were examined with O:19 strains being distinguishable from non-O:19 strains in each case. Non-serotype O:19 strains tested included: O:1, O:2, O:6, O:7, O:8, O:11, O:15, O:16, O:17, O:23, O:25, O:36, O:38, O:50, O:64 and four non-typable strains.

5.1.2 Serotype O:19 PCR method

In our hands, the O:19 PCR test as described by Misawa, Allos and Blaser (1998) did not detect the O:19 control strain. The method was therefore modified by optimising the primer, magnesium chloride concentration and increasing amplification annealing temperatures, such that the assay was able to detect control strains before commencing large scale testing of our isolates. Following completion of our testing, an *erratum* was published by Misawa, Allos and Blaser (2000) in the same journal 2 years later stating that primer concentration was incorrectly published in their original paper.

In our study, crude bacterial DNA preparations were prepared by heating a MacFarland 1.0 suspension of bacteria (equivalent to a transmission of 70% as determined by a Vitek colorimeter), to 95°C for 10 minutes, after which the suspension was cooled to 4°C and used immediately for PCR. The optimised reaction mixture for PCR and the thermal cycling conditions using the Perkin Elmer DNA Thermal Cycler Model 9600 are given in Tables 5.1 and 5.2 respectively using primer sequences as given in Table 2.3: Details of primers chosen and designed.

The combined program outlined in Table 5.2, was originally designed by Misawa, Allos and Blaser (1998) to eliminate non-specific fragments being produced by non-O:19 strains. This program originally had an annealing temperature in the first round PCR of 50°C and an annealing temperature of 48°C in the second round PCR. Using these conditions a fragment of the correct size was still observed when testing the O:19 negative control strain (serotype O:3). To eliminate this problem, the annealing temperatures were increased to 52°C and 50°C

Table 5.1: Optimised reaction conditions for serotype O:19 PCR assay

Reagent	Final Concentration
10 X PCR Buffer II	1 X
Magnesium Chloride (mM)	4.65
*Gyr*B Sense Primer (μM)	0.7
*Gyr*B Anti-sense Primer (O:19)	0.7
dNTPs (dATP, dGTP, dCTP, dUTP)	200 μM of each
AmpliTaq® Gold DNA Polymerase	0.5 U
Water	Volume adjusted to 50 μL

Table 5.2: Optimised amplification conditions for serotype O:19 PCR assay

Amplification Step	Conditions
DNA Polymerase Enzyme activation (*AmpliTaq*® Gold DNA Polymerase)	10 minutes, 94°C
10 cycles – Denaturation	1 minute, 94°C
Annealing	1 minute, 52°C
Extension	1 minute, 72°C
30 cycles – Denaturation	1 minute, 94°C
Annealing	1 minute, 50°C
Extension	1 minute, 72°C

respectively. Furthermore, the PCR was repeated on all isolates to detect non-O:19 *Campylobacter* using the GyrB Antisense (non-O:19) primer as given in Table 2.3: Details of primers chosen and designed. This PCR was performed with the same reaction mixture (Table 5.1) and optimised thermal cycling conditions (Table 5.3).

Extracted DNA from ATCC 700297 (serotype O:19) and ATCC 43431 (serotype O:3) was used as positive/negative controls in the O:19 PCR and negative/positive controls in the non-O:19 PCR.

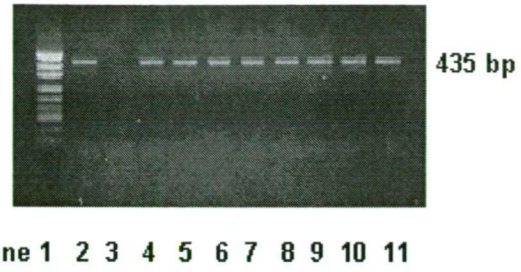
Table 5.3: Optimised amplification conditions for non-serotype O:19 PCR assay

Amplification Step	Conditions
DNA Polymerase Enzyme activation (*AmpliTaq*® Gold DNA Polymerase)	10 minutes, 94°C
40 cycles – Denaturation	1 minute, 94°C
Annealing	1 minute, 54°C
Extension	1 minute, 72°C

5.1.3 Serotype O:19 PCR results

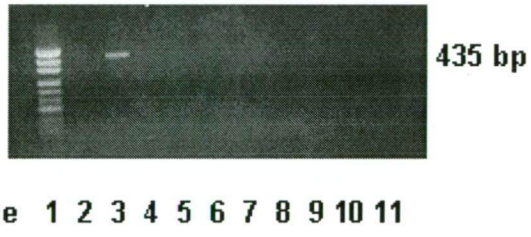
Of the 250 strains of *Campylobacter* tested at the RHH, 8 (3.2%) produced a positive O:19 PCR result (Figure 5.1) and were also negative by the non-O:19 PCR (Figure 5.2). However, when these O:19 positive isolates were sent to a reference laboratory, Melbourne Diagnostic Unit -University of Melbourne (MDU) for serotype confirmation, 3 strains were found to be Penner serotype O:18 with the remainder being non-typable. Due to the possibility of the PCR cross-reacting with serotype O:18, 7 further strains of serotype O:18 were obtained from the reference laboratory for testing by the O:19 PCR. Unlike the 3 serotype O:18 strains identified in our study, all of the serotype O:18 strains obtained from MDU produced negative O:19 PCR results and a positive non-O:19 PCR. While only a small number of serotypes were

Figure 5.1: PCR to detect Serotype O:19 *Campylobacter* – agarose gel electrophoresis stained with ethidium bromide



Lane 1: MWM, Lane 2: Positive control (serotype O:19) *C. jejuni*, Lane 3: Negative control (serotype O:3) *C. jejuni*, Lane 4-11: Tasmanian gastroenteritis strains of *C. jejuni*

Figure 5.2: PCR to detect non-Serotype O:19 *Campylobacter* – agarose gel electrophoresis stained with ethidium bromide



Lane 1: MWM, Lane 2: Negative control (serotype O:19) *C. jejuni*, Lane 3: Positive control (serotype O:3) *C. jejuni*, Lane 4-11: Tasmanian gastroenteritis strains of *C. jejuni*

tested in the original paper (Misawa , Allos and Blaser, 1998) and these did not include O:18 strains, our results prove that other serotypes may produce a positive reaction with this PCR which should thus only be used as a preliminary screening tool before being serotyped by the Penner system. Furthermore, the original PCR method only tested *C. jejuni* isolates. To exclude the possibility of false positive PCR results for other *Campylobacter* species, the O:19 PCR and non-O:19 PCR were repeated on all non-*C. jejuni* isolates found in our study. All 13 non-*C. jejuni* isolates tested produced a negative result for both the O:19 and non-O:19 PCR; a feature of this PCR test not reported in the original paper (Table 5.4).

Table 5.4: Serotype O:19 and non-O:19 PCR results for *Campylobacter* isolates

Number of isolates	O:19 PCR	Non-O:19 PCR	Identification
8	POSITIVE	NEGATIVE	*C. jejuni* subsp. *jejuni*
229	NEGATIVE	POSITIVE	*C. jejuni* subsp. *jejuni*
13	NEGATIVE	NEGATIVE	10 *C. coli* 2 *C. lari* 1 *C. upsaliensis*

5.2 *Cholera* Toxin Binding Inhibition EIA

5.2.1 Background

Patients presenting with GBS are found to possess a high prevalence of autoantibodies against the monoganglioside Gm1 present on peripheral neurons (Schwerer *et al*, 1995). Gm-like epitopes have also been detected in the lipopolysaccharide (LPS) of many GBS-associated strains of *C. jejuni* (Aspinall *et al*, 1994b). It has therefore been suggested that infection with these strains of *Campylobacter* may stimulate an autoimmune response against peripheral nerves (Griffin *et al*, 1996). An ELISA was developed by Sack *et al,* (1998) to detect the presence of Gm1 in the LPS of *Campylobacter* strains.

The assay was based upon the ability of Gm1 ganglioside to bind to *Cholera* toxin (Ctx). A pre-incubation step of *Campylobacter* LPS with Ctx binds Gm1-like epitopes in the LPS, therefore blocking available antibody binding sites, thus producing a negative result in the assay. With this assay, Sack *et al* (1998) found 27 of 118 (23%) *C. jejuni* subsp. *jejuni*

isolates, 1 of 5 (20%) *C. coli* isolates and 9 of 17 (53%) *Helicobacter pylori* isolates tested positive.

Other bacteria tested including 34 isolates of various non-*C. jejuni/C. coli* campylobacters and 18 *Escherichia coli* isolates, produced negative results. In another study, Lastovica and Sack (1999) also detected Gm1 epitopes in one isolate *of Campylobacter mucosalis* , two isolates of *C. jejuni* subsp. *doylei* and one *Helicobacter acinonyx*. In this study, two *C. jejuni* isolates associated with GBS were found to be Gm1 negative. Diker and Hascelik (1999), performed Gm1 analysis upon 90 animal strains of *Campylobacter*. They found 21% of strains were Gm1 positive and were found in a variety of hosts including chickens, sheep, dogs and cattle.

5.2.2 Method

Testing was performed as given in Section 2.7: *Cholera* Toxin Inhibition EIA for the detection of Gm1 epitopes on bacterial cell walls.

5.2.3 Results

In our study, 87 of 237 (37%) *C. jejuni* subsp. *jejuni* isolates were positive, with all 10 isolates of *C. coli*, 2 isolates of *C. lari* and 1 isolate of *C. upsaliensis* producing a negative result (Table 5.5) and 10 of 10 (100%) GBS-associated *Campylobacter* strains testing positive (Table 5.6). The assay was easy to perform and all positive strains showed a decrease of greater than 90% of the OD relative to the negative control (Figure 5.3).

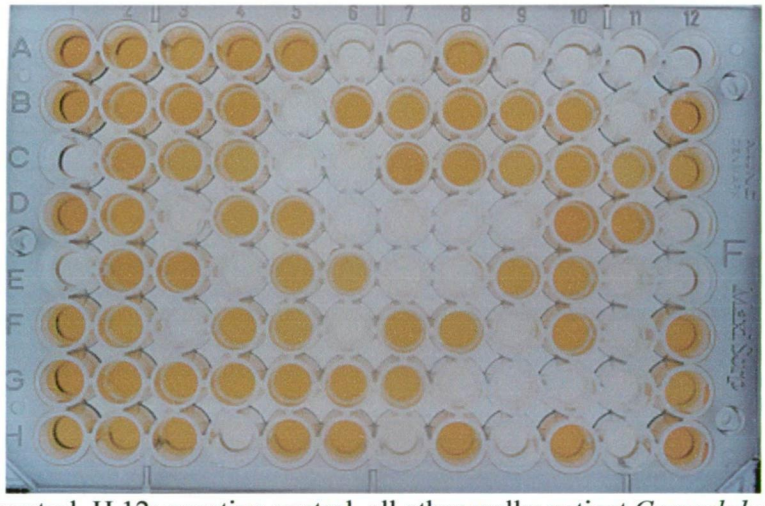
Table 5.5: Gm1 positive rates: *Cholera* Toxin Binding Inhibition Assay

Number of isolates	*Cholera* toxin binding Inhibition EIA	Identification
87	**POSITIVE**	***C. jejuni* subsp. *jejuni***
163	**NEGATIVE**	**150 *C. jejuni* subsp. *jejuni*** **10 *C. coli*** **2 *C. lari*** **1 *C. upsaliensis***

5.2.4 Discussion

The relatively common occurrence of the Gm1 epitope found in the Tasmanian *Campylobacter* isolates and the low incidence of GBS within the Tasmanian community

<u>Figure 5.3</u>: Detection of Gm1 epitopes in *Campylobacter* species by the *Cholera* Toxin Binding Inhibition Assay



H 11: positive control, H 12: negative control, all other wells: patient *Campylobacter* strains

suggests that other factors such as host genetic predisposition and/or the presence of undetermined bacterial virulence factors are important in the development of GBS. Furthermore, other researchers have found that non-Gm1 positive *C. jejuni* strains associated with GBS possess other ganglioside like epitopes in their core LPS (Nachamkin *et al*, 1999). While this adds weight to the molecular mimicry theory, it does not aid researchers in the detection of potentially neuropathic strains of *Campylobacter* before the onset of GBS. It is therefore imperative to find more specific markers for the detection of these strains.

Table 5.6: GBS control strains of *C. jejuni*

STRAIN	COUNTRY OF ORIGIN	SEROTYPE (Penner)	Ctx	PFGE type	MLST type *
HB 9529 (GBS1)	China	O:19	+	1	22
HB 9313 (GBS2)	China	O:19	+	1	22
HB 9643 (GBS3)	China	O:19	+	1	22
INP 26 (GBS4)	Mexico	O:19	+	1	22
INP 8 (GBS5)	Mexico	O:19	+	1	22
ATCC700297 (GBS6)	China	O:19	+	1	22
SDY (GBS7)	Australia	NT	+	1	NT
28134:94 ASM (GBS8)	South Africa	O:41	+	4	5
6491 AZR (GBS9)	Unknown	O:23	+	5	42
260:94 RXM (GBS10)	South Africa	O:41	+	3	362
DJ5 (TASGBS)	Australia	NT	+	2	525

Ctx: *Cholera* Toxin Binding Inhibition Assay, PFGE: Pulsed Field Gel Electrophoresis, MLST: Multi Locus Sequence Typing. NT: Not tested, +: positive.
* MLST results provided by Dr. Dingle, The Peter Medawar Building for Pathogen Research and Department of Zoology, Oxford University, England.

5.3 Pulsed Field Gel Electrophoresis

5.3.1 Background

Neither the Gm1 typing of *Campylobacter*, nor the detection of serotype O:19 isolates by PCR has provided scientists with a definitive testing method to detect strains of

neuropathogenic campylobacters. One aim of this project was to determine if a genetic based bacterial typing method could differentiate community gastrointestinal isolates of *Campylobacter* found in Tasmania from strains isolated from GBS patients, something that the phenotypic methods were not able to do. The typing method chosen for this purpose was PFGE.

5.3.2 Method

Testing was performed as described in Section 2.9: Pulsed Field Gel Electrophoresis.

5.3.3 Results

Clustering of GBS-associated strains by PFGE can be seen when compared with the ten most predominant strains seen in Tasmania (Figure 5.4). Interestingly, a Tasmanian GBS-associated strain of *C. jejuni* which was recently isolated and not part of the original 250 strains studied, grouped with the Tasmanian strains rather than the GBS strains. A review of this patient showed that this was clearly a case of GBS. The clustering of patterns may therefore be a clonal effect in a geographically isolated area, such as Tasmania, rather than the uniqueness of GBS strains as the GBS type strains were obtained from an overseas source.

5.3.4 Discussion

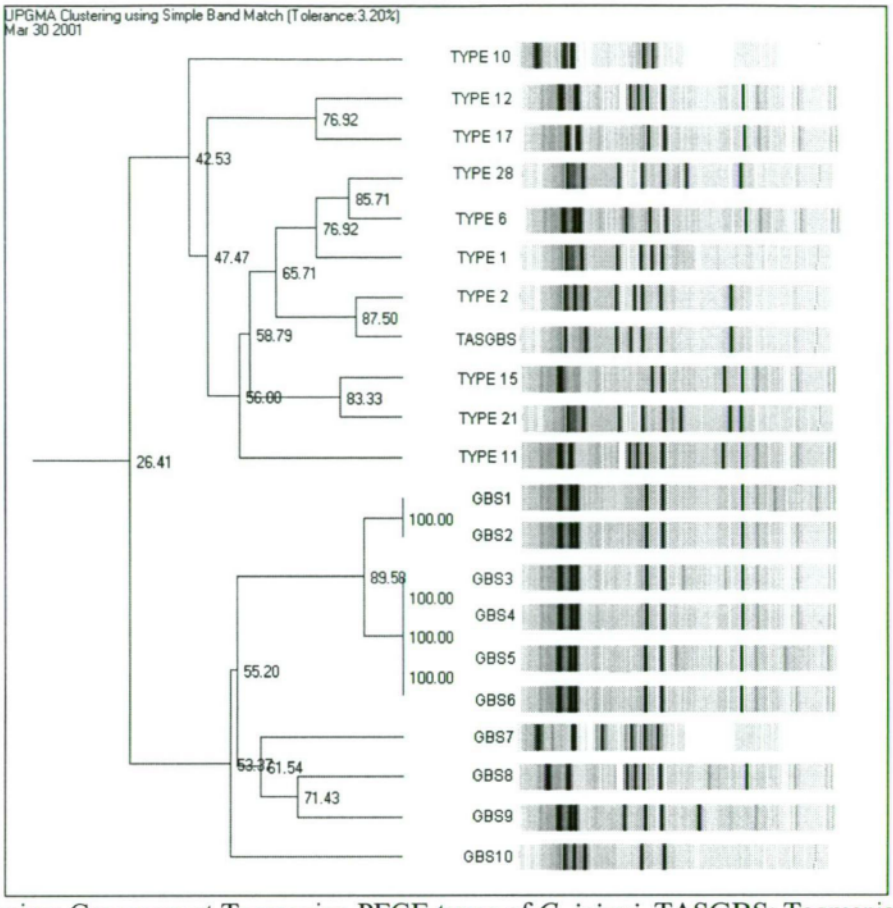
In this study, the similarity amongst the serotype O:19 GBS strains (GBS1 – 6) can clearly be seen and variable patterns seen with the other serotypes studied (GBS8 & GBS10: serotype O:41, GBS 9: serotype O:23) when using PFGE, MLST typing systems (Table 5.6). This clonal nature of certain serotypes has been previously reported. Molecular characterization of *C. jejuni* associated with GBS using amplified fragment length polymorphism (AFLP) and flagellin typing of 10 serotype O:41strains showed a marked degree of homology (Wassenaar, T.M., et al, 2000) as did serotype O:19 (Nishimura *et al,* 1997). Other serotypes such as O:1 and O:2 on the other hand have shown to be quite genetically heterogeneous as a group (Fayos *et al*, 1993).

As a group, the GBS strains did not have distinctive PFGE banding or patterns that differentiated them from non-GBS isolates. This is in agreement with a study of 18 GBS and MFS strains of *C. jejuni* (Endtz *et al*, 2000) in which analysis by AFLP, PFGE, *fla*A polymorphism and randomly amplified polymorphic DNA (RAPD) showed no clustering effect when compared with non-GBS/MFS strains and no unique molecular markers.

5.4 Summary

Current knowledge of GBS associated strains of *Campylobacter* show that they, as a group have a number of attributes in common. Firstly, certain serotypes are isolated from GBS

Figure 5.4: PFGE dendrogram analysis of GBS associated and Tasmanian community acquired *Campylobacter* isolates following digestion with *Sma*I



TYPE Strains: Commonest Tasmanian PFGE types of *C. jejuni,* TASGBS: Tasmanian GBS strain of *C. jejuni*, GBS1-GBS10: GBS strains of *C. jejuni* from various countries

patients at an increased frequency compared to other serotypes. However, not all patients who are infected with these serotypes develop GBS. Secondly, GBS associated strains have cell wall structures that mimic human gangliosides such as Gm1. However not all *Campylobacter* enteritis patients with Gm1 positive strains develop GBS (prevalence rates for Gm1 positive strains and GBS patients in Tasmania; 37.2 and 2.3 per 100,000 population respectively). Genetic epidemiological techniques such as PFGE have the ability to differentiate isolates into a number of subgroups as determined by their restriction digest profiles and hopefully to be able to separate GBS isolates from those that cause enteritis. However this was not to be the case as no distinctive profiles or bands were found using this technique. Other genetic studies which also look at a small number of genetic regions, such as variation in the flagellin region (*fla*A polymorphism), have also not shown distinctive subgrouping that mark potentially neuropathogenic strains. Therefore, a definitive marker has not yet been found to identify GBS associated strains from others that cause enteritis.

It was hypothesised that GBS isolates of *Campylobacter* not only require certain attributes as described above but also require a further unknown pathogenic determinant which is not present in all Gm1 positive strains and all members of serotypes associated with GBS. Characterisation of this pathogenic determinant might therefore provide the information necessary to understand the aetiology of GBS development and develop screening to identify such strains. Comparative genomic methods, such as subtractive hybridisation offer ways to investigate subtle differences in the genetic makeup of strains of bacteria.

CHAPTER 6

IDENTIFICATION OF GENOMIC SPECIFIC DNA SEQUENCES IN GBS RELATED STRAINS OF *C. jejuni*

6.1: Introduction

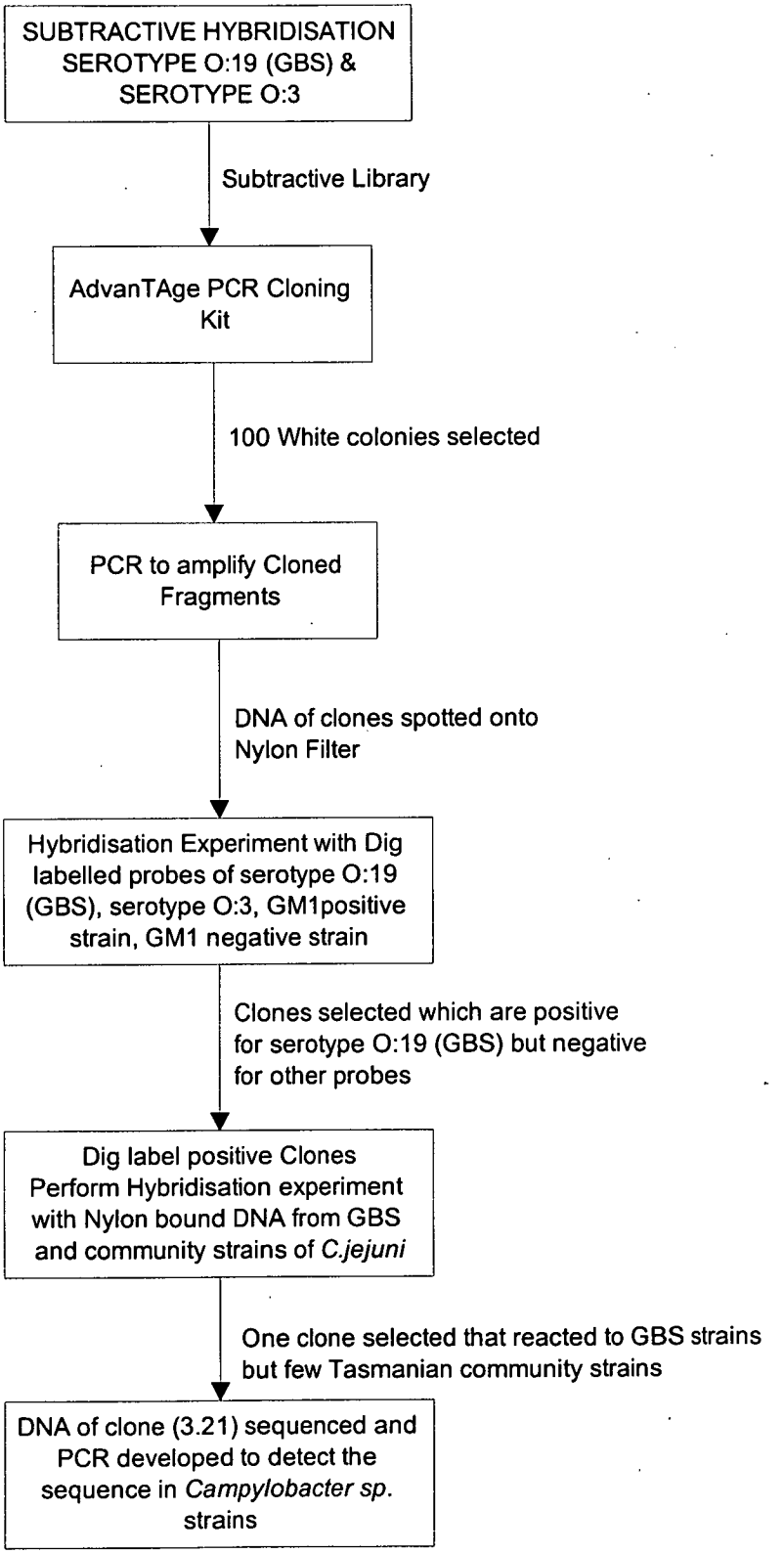
It has been known since 1993 that some strains of *Campylobacter jejuni* synthesise sialic acid and have sialylated carbohydrate residues in the lipopolysaccharide of their cell walls that are identical to human gangliosides. The "molecular mimicry" hypothesis put forward by some researchers suggests that these strains elicit an autoimmune response (Griffin *et al,* 1996). The evidence from various studies however shows many discrepancies. For example, *C. jejuni* containing Gm1 like epitopes have been isolated from some GBS patients but these same patients do not demonstrate detectable levels of serum antibodies to Gm1 (Hao *et al,* 1998). Furthermore, anti-Gm1 antibodies were detected in only 2 of 6 GBS patients infected with a clonally indistinguishable Penner O:41 strain of *C. jejuni* (Gregson and Lastovica, 1999). In addition, sialylated lipo-oligosaccharides are found in other pathogenic bacterial genera including *Neisseria* and *Haemophilus* but these organisms have not been shown to be associated with GBS. Current evidence therefore suggests that molecular mimicry between cell wall sialylated carbohydrate residues in *C. jejuni* and human gangliosides cannot solely account for *C. jejuni* associated GBS. Other factors may play an important role in the pathogenesis of GBS, such as host susceptibility, molecular mimicry of molecules other than the lipopolysaccharide, the presence of a currently unknown bacterial virulence factor or a combination of any of these factors. The aim of this section of the study was to investigate the last point by examining GBS-associated strains of *C. jejuni* for unique DNA regions, not associated with Gm1 expression in order to identify other important molecules in the pathogenesis of GBS. Subtractive hybridisation techniques were used to locate specific DNA sequences present in the genome of a strain of *C. jejuni* that has been associated with GBS development (Penner serotype O:19) that were not present in the genome of a strain of *C. jejuni* from a serotype never isolated from a GBS patient (Penner serotype O:3). An overview of the experimental approach is given in Figure 6.1

6.2: Subtractive Hybridisation

6.2.1 Background

Subtractive hybridisation is a powerful technique for the isolation of genes present in one bacterial cell population but absent in another and is based upon a process called driver excess hybridisation. Basically, the genomic DNA from the organism that potentially contains genes of interest (the tester) is hybridised to complementary genomic DNA that should lack these sequences (the driver). The driver DNA is present at a much higher concentration than the

Figure 6.1: Detection of a GBS specific DNA sequence in *C. jejuni*



tester DNA (at least 10:1) and this dictates the speed of the reannealing reaction. The tester and driver nucleic acid populations are allowed to hybridise and sequences common to both populations form hybrids. Following hybridisation, both the tester-driver hybrids and excess unhybridised driver are removed leaving behind tester DNA that is enriched with tester specific DNA sequences. This DNA can then be used to prepare a library enriched in tester-specific clones or to make a probe that can be used to screen a library for tester-specific clones.

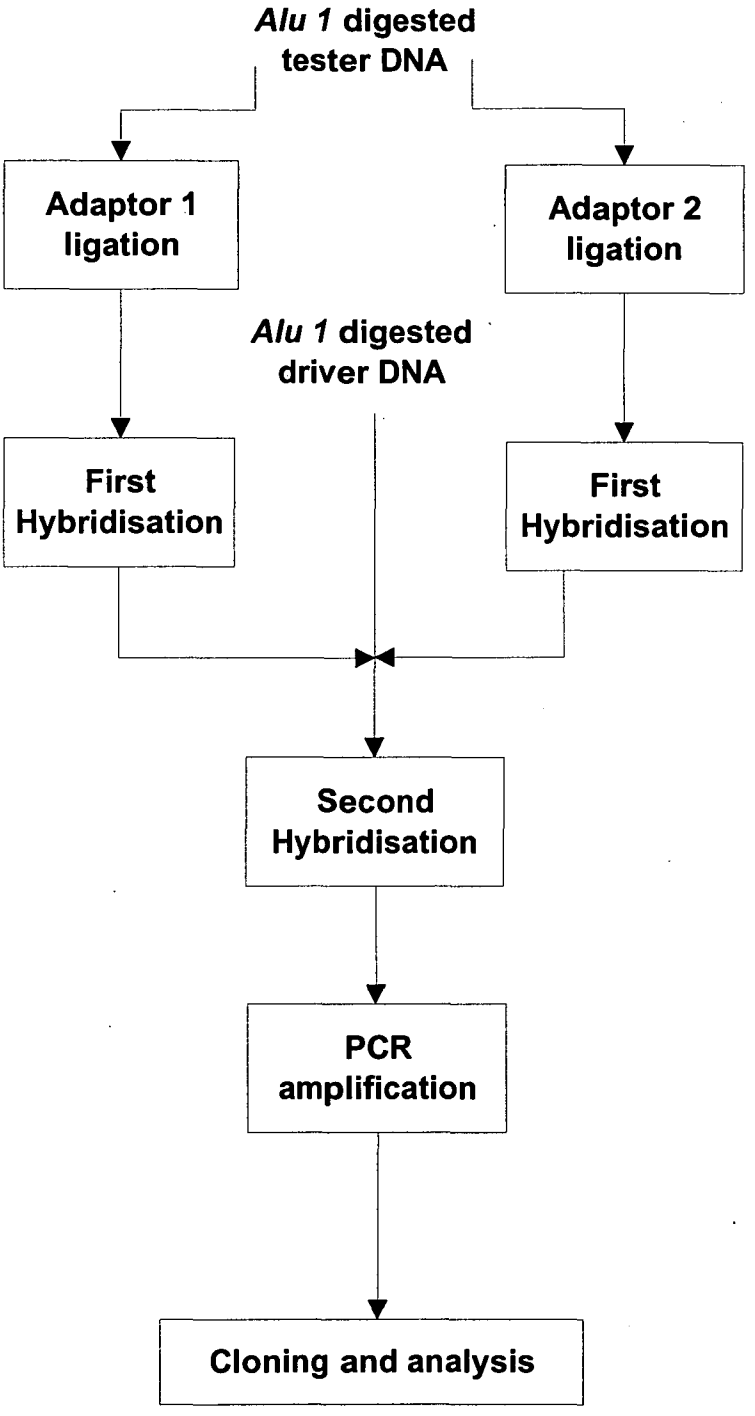
This approach has been used for the identification of virulence factors in uropathogenic strains of *E. coli* (Janke *et al*, 2001). Furthermore, the genome of *Burkholderia pseudomallei* has also been subtracted against the closely related avirulent species *Burkholderia thailandensis* producing a number of DNA sequences specific for *B. pseudomallei* (Brown and Beacham, 2000) encoding putative virulence factors. The usefulness of this technique has also been utilised in the plant kingdom with studies on *Brassica napus* (Buchanan-Wollaston and Ainsworth, 1997), the animal kingdom on cloning of carp (*Cyprinus carpio*) C-type lectin (Fujiki *et al*, 2001) and in human tumour cell studies (Beyer-Sehlmeyer *et al*, 1999).

6.2.2 Method

Testing was performed as given in Section 2.10.1: Subtractive hybridisation – DNA subtraction.

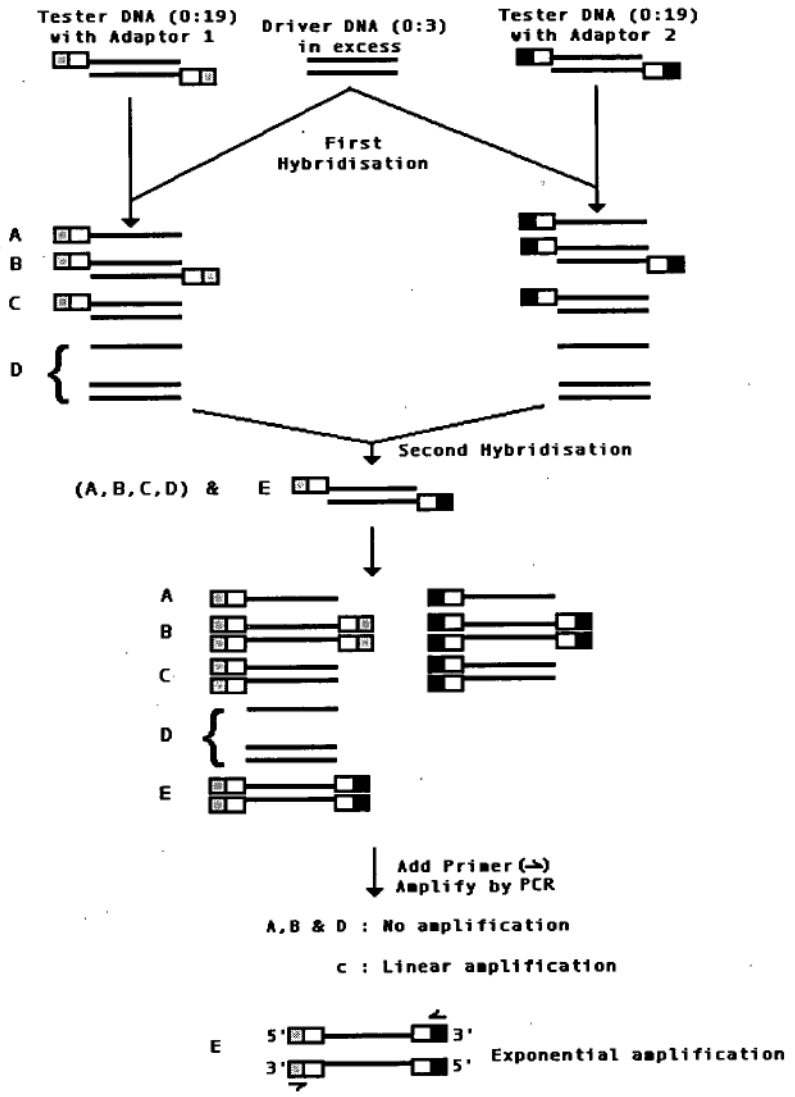
In this study, the tester DNA was extracted from a GBS-associated *Campylobacter jejuni* (ATCC 700297) and the reference driver DNA from an ATCC 43431 strain, *Campylobacter jejuni* serotype O:3 (a serotype not known to be associated with GBS). The CLONTECH PCR-Select™ Bacterial Genome Subtraction Kit was used to obtain the subtracted DNA library and a generalised overview of the experimental process can be seen in Figure 6.2. Briefly, the genomic DNAs of the two bacterial strains being compared (1.5 – 2 μg of DNA per subtraction) were digested with *Alu*I. The tester DNA was then subdivided into two portions, each of which was ligated to a different adaptor. Excess driver DNA was then added to both aliquots and allowed to hybridise. The resultant mixtures were heat denatured and then cooled to anneal, generating molecules A, B, C and D as outlined in Figure 6.3. The two primary hybridisation samples were then pooled. Only single stranded tester DNA sequences that are not present in the driver DNA can hybridise at this stage producing a double stranded DNA molecule with different adaptors on each end (Molecule E, Figure 6.3). Following the ends of the molecules being filled in by DNA polymerase, a PCR was performed using primers complimentary to the adaptor sequences. Only type E molecules were amplified exponentially to produce a mixture of tester specific DNA sequences. Next a

Figure 6.2: Overview of the CLONTECH PCR-Select™ Subtractive Hybridisation Procedure



Adapted from CLONTECH PCR-Select™ Bacterial Genome Subtraction Kit User Manual (PT3170-1)

Figure 6.3: DNA molecule structures found at various stages of subtractive hybridisation



Adapted from CLONTECH PCR-Select™ Bacterial Genome Subtraction Kit User Manual (PT3170-1)

secondary PCR amplification was performed using nested primers to further reduce any background PCR products and enrich tester-specific sequences (Figure 6.4).

6.3: Cloning of subtractive hybridisation enriched DNA

6.3.1 Method

The cloning and transformation protocol was as given in Section 2.10.2: Subtractive hybridisation – Cloning.

Cloning of the subtractive library was performed using the AdvanTAge™ PCR Cloning Kit from CLONTECH Laboratories, Inc. The PCR products enriched with tester-specific sequences from the subtractive hybridisation experiment was ligated into the pT-Adv 3.9 kb cloning vector without prior purification (Figure 6.5). Ligation of products was performed overnight at 14°C into cloning vector followed by transformation into TOP10F' *Escherichia coli* cells and plated onto LB/X-gal/IPTG agar plates containing 50 µg/ml of kanamycin. Transformants were screened using kanamycin resistance and β-galactosidase α-complementation testing (blue/white screening). White colonies growing on the kanamycin containing media should contain inserts and were screened further (Figure 6.6).

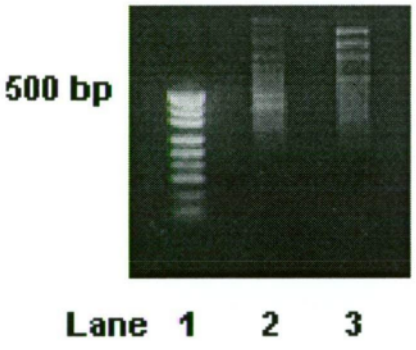
6.4: Screening cloned DNA

6.4.1 Method

One hundred individual positive clones were selected and grown overnight on LB plates containing kanamycin. As the cloned DNA still had the adaptor DNA attached from the hybridisation experiment, these adaptor sequences were used as targets to amplify the fragments to obtain concentrated DNA. A 0.5 MacFarland suspension was made of each clone and incubated at 95°C for ten minutes. The sample was subsequently cooled and then used in a PCR with the nested primers 1 and 2R according to the methods outlined in the CLONTECH PCR-Select™ Bacterial Genome Subtraction Kit. This produced concentrated DNA from the cloning site of each individual clone of *E. coli* which was then bound to Hybond™ -N+ nylon nucleic acid transfer membrane. Dot blot hybridisation was then performed as given in Section 2.8 Dot Blot Hybridisation and probed sequentially with digoxigenin labeled probes (DIG DNA Labeling Kit - Boehringer Mannheim) made from DNA digested with restriction enzyme *Alu*I of the following four strains; *Campylobacter* strains serotype O:19 (GBS strain), serotype O:3, a Tasmanian Gm1 positive isolate and a Tasmanian Gm1 negative isolate at concentrations of 25 ng/mL. An anti-digoxigenin-alkaline phosphatase conjugate was used at a dilution of 1:5000 in 1% blocking solution.

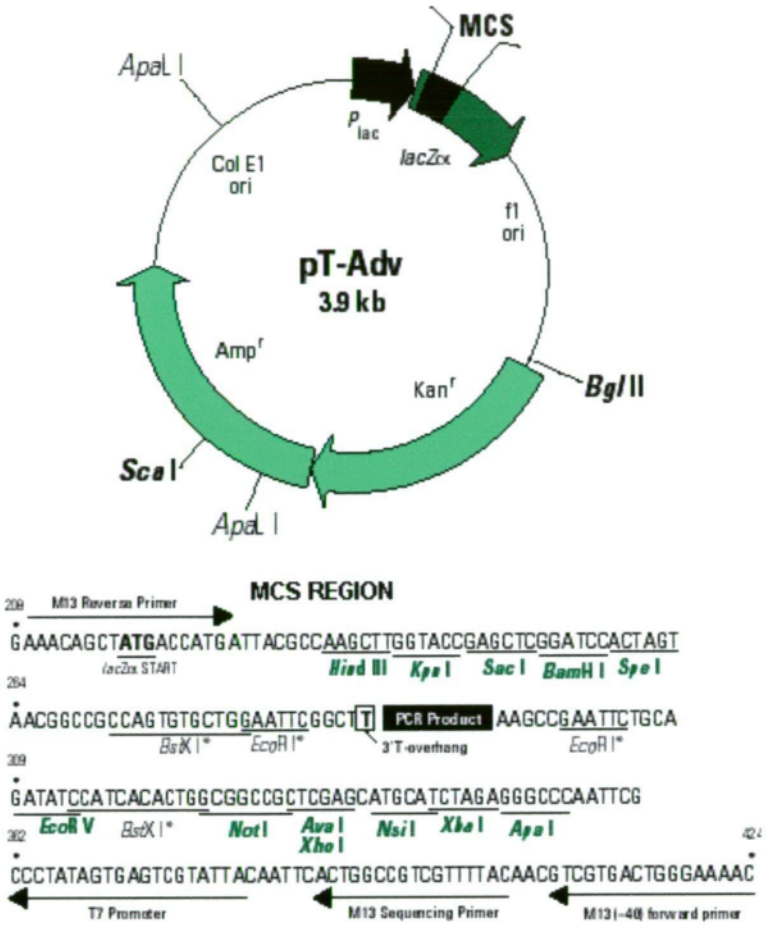
DNA samples were selected that hybridised at 45°C to the serotype O:19 probe but not to the other three probes.

Figure 6.4: Subtractive hybridisation library of serotype O:19 specific DNA sequences



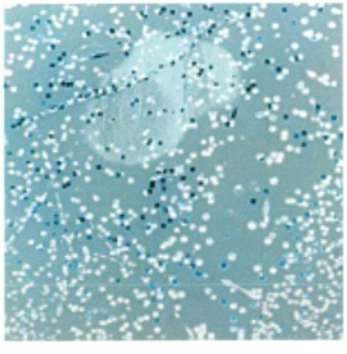
DNA species remaining after subtraction of common DNA sequences. This will contain DNA sequences that are unique to the O:19 serotype *C. jejuni*.
Lane 1: Molecular weight marker, Lane 2: *Campylobacter* sp. subtractive library, Lane 3: *E.coli* control subtractive library. 2% agarose gel stained with ethidium bromide.

Figure 6.5: Restriction Map and Multiple Cloning Site (MCS) of pT-Adv Cloning vector



Unique restriction sites are in bold. Restriction sites with asterisks (*) are present only in the MCS and can be used to excise the inserted PCR product.
Source: BD Bioscience: CLONTECH web site: http://www.clontech.com/techinfo/vectors

Figure 6.6: AdvanTAge PCR cloning experiment



Screening of cloned library, seen in Figure 6.4, for recombinant plasmids.
LB/X-gal/IPTG & kanamycin agar plates showing blue and white colonies (no DNA inserts and inserts respectively)

Each DNA sample with the above criteria was then digoxigenin labeled and used as a probe in individual dot blot hybridisation experiments against purified DNA from strains of *Campylobacter jejuni* isolated from patients with (as given in Table 5.6) and without GBS.

6.4.2 Results

One clone was detected (Clone 3.21) that reacted with DNA from 100 % (11/11) of GBS isolates but only 14.5% (7/48) of the Tasmanian community strains of *C. jejuni* tested. This DNA was sequenced as given in Section 2.12: DNA sequencing to provide 559 b.p. sequence (Figure 6.7). The Genbank database was searched for comparisons with the data from the sequencing analysis to determine any previously described identities for this sequence. Genbank DNA matches are shown in Figure 6.8 with the highest level of homology found to be to a 138 nucleotide sequence from *C. jejuni* subsp *jejuni* NCTC 11168. This region corresponds to designated gene "Cj1013c", a probable membrane protein of undefined function (Figure 6.9) as determined by the *C. jejuni* sequencing data held at the Sanger Centre and available on the World Wide Web (URL: http://www.sanger.ac.uk/Projects/C_jejuni/).

6.5: Clone 3.21 PCR

6.5.1 Method

The sequence information for clone 3.21 was used to identify regions of DNA suitable for amplification by PCR. Designed primers were compared for specificity against the Genbank database using the computer program Basic BLAST (NCBI web site).

The optimised reaction mixture for PCR and the thermal cycling conditions using the Perkin Elmer DNA Thermal Cycler Model 480 are given in Tables 6.1 and 6.2 respectively.

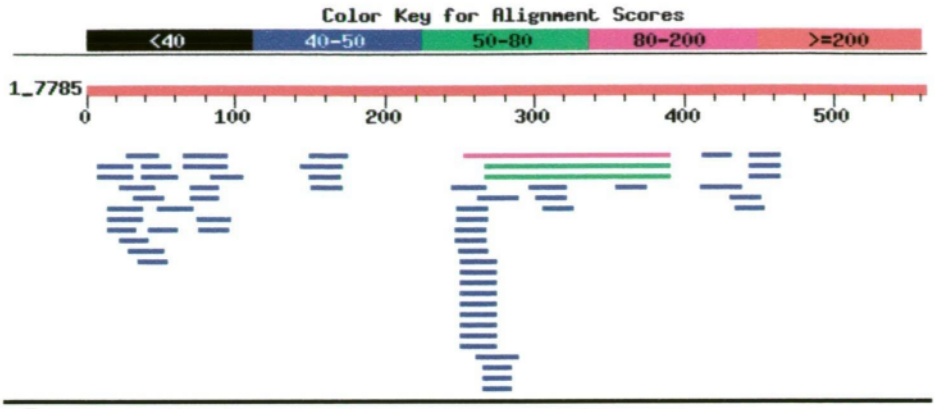
Table 6.1: Optimised reaction conditions for PCR assay (3.21)

Reagent	Final Concentration
10 X PCR Buffer II	1 X
Magnesium Chloride (mM)	1.65
3.21 Sense Primer (μM)	0.45
3.21 Anti-sense Primer (μM)	0.45
dNTPs (dATP, dGTP, dCTP, dUTP)	200 μM of each
AmpliTaq® Gold DNA Polymerase	0.5 U
Sample	100 ng of genomic DNA
Water	Volume adjusted to 50 μL

Figure 6.7: Sequence data of cloned DNA fragment from Clone 3.21

GCAATNCTATCATCAAAATGCAAATTTCTTTTAATAATGAAAGCA
AAGAAATTTTACTTTTTNCAAATTACAACAATGAAAATGTTTTAC
CTTTTAGATTAGGAGGTGAAATTTTTGCACTCAACTGGGGTCCTG
AAGAGATTAAACTGCCTTTTTCTTTGGCTTTAAAAGATTTTATCCT
TGATCGCTACGCAGGTTCTATGAGTCCTTCTTCTTATGCTTCAGAT
ATCGAAGTGATCGATCAAGATAAAAGTTTTGAGTATAAAATTTTT
ATGAATAATGTTTTAGATTATGGCGGATATAGATTTTTTCAAAGT
TCTTATGATCAAGACGAACAAGGCACCATACTTTCAGTCAATAAA
GATCCTGGAAAAATTCCAACTTATATAGGCTATACTTTGCTGACT
TTAGGTTTTTTATGGGATTTTATTTGCTAAAAACTCAAGATTTCAA
AAATTATCAAACTATCTTAAAAATCAAAAAAATCTTTTATTAATT
CTTTTTTGNCTTTTTGCTTTTAACATAAAAAAGGTTTTGCCCGATG
AAAACACCTTAAAGACCTC

Figure 6.8: Genbanks analysis of clone 3.21 DNA




```
Score     E
Sequences producing significant alignments:                          (bits) Value

gi|6968444|emb|AL139077.2|CJ11168X4  Campylobacter jejuni su...    123    2e-25
gi|2313475|gb|AE000554.1|AE000554  Helicobacter pylori 26695...     50    0.003
gi|4155575|gb|AE001528.1|AE001528  Helicobacter pylori, stra...     50    0.003

Alignments

>gi|6968444|emb|AL139077.2|CJ11168X4   Campylobacter jejuni subsp.
jejuni NCTC 11168 complete genome;
            segment 4/6
          Length = 282183

Score =  123 bits (62), Expect = 2e-25
Identities = 119/138 (86%)
Strand = Plus / Minus

Query: 254  ttttgagtataaaatttttatgaataatgttttagattatggcggatatagatttttca 313
            |||||| |||| ||||||||||||||||||||||||||||  || ||||||||| |||
Sbjct: 5917 ttttgattatagaatttttatgaataatgttttagattatgatggctatagatttatca 5858


Query: 314  aagttcttatgatcaagacgaacaaggcaccatactttcagtcaataaagatcctggaaa 373
            |||||||||||||||||| ||| |||| ||  |  | || || |||||||||| || ||
Sbjct: 5857 aagttcttatgatcaagatgaaaaagggactgttttatctgtaaataaagatccgggcaa 5798


Query: 374  aattccaacttatatagg 391
            ||| ||||||||||||||
Sbjct: 5797 aataccaacttatatagg 5780
```

Source: Standard nucleotide-nucleotide BLAST [blastn] search at the National Center for Biotechnology Information (NCBT) (http://www.ncbi.nlm.nih.gov)

Figure 6.9: Probable gene function of Clone 3.21 DNA match at Genbank

ORGANISM Campylobacter jejuni subsp. jejuni NCTC 11168 Bacteria; Proteobacteria; epsilon subdivision; Campylobacter group; Campylobacter.
gene complement(3961..7206) /gene="Cj1013c" CDS complement(3961..7206) /gene="Cj1013c" /note="Cj1013c, probable membrane protein, len: 1081 aa; contains three domains; aa 1-90 contains three membrane spanning domains; aa 90-780 is non-membrane, and aa 780-1081 contains ten possible membrane spanning
Source: *C. jejuni* sequencing data at the Sanger Centre (http://www.sanger.ac.uk/Projects/C_jejuni/).

Table 6.2: Optimised amplification conditions for PCR assay (3.21)

Amplification Step	Conditions
DNA Polymerase Enzyme activation (*AmpliTaq*® Gold DNA Polymerase)	5 minutes, 94°C
36 cycles – Denaturation	30 seconds, 94°C
Annealing	30 seconds, 56°C
Extension	1.5 minutes, 72°C

6.5.2 Results

The resultant PCR experiment with purified *C. jejuni* DNA produced the expected 162 bp fragment (Figure 6.10) in 10 of 11 (91%) GBS isolates of *C. jejuni* and only 6 of 57 (10.5%) Tasmanian enteritis isolates of *C. jejuni* (Table 6.3).

PFGE analysis of these isolates showed the 57 Tasmanian isolates could be subdivided into 27 different profiles with the 6 PCR positive isolates belonging to 5 of these.

Gm1 analysis of the 57 Tasmanian strains tested showed the 3.21 gene could be detected in only 5% of Gm1 positive isolates and 15.6% of Gm1 negative isolates (Table 6.4).

Table 6.3: PCR 3.21 results for GBS associated isolates and Tasmanian enteritis isolates of *C. jejuni*

PCR	GBS isolates	Tasmania enteritis isolates
3.21	10/11 POSITIVE	6/57 POSITIVE

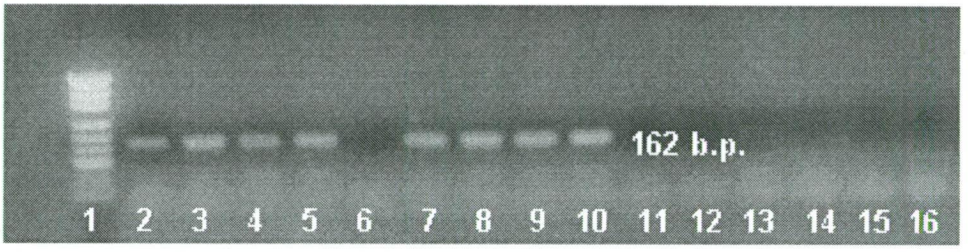
Table 6.4: Gene 3.21 PCR/Gm1 analysis of Tasmanian *C. jejuni* isolates

	Positive for Gm1	Negative for Gm1
PCR Positive for 3.21	**1**	**5**
PCR Negative for 3.21	**19**	**32**

6.5.3 Discussion

This confirms that the 3.21 gene is not associated with the Gm1 gene but could still be associated with some other sialylated carbohydrate residue in the lipopolysaccharide or other

Figure 6.10: PCR For Gene 3.21 – agarose gel stained with ethidium bromide



LANE 1: Molecular Weight Marker, LANES 2-10: GBS strains of *C. jejuni*, LANES 11-16: Tasmanian clinical isolates of *C. jejuni*

unrelated protein structure. As 91% of GBS-associated *Campylobacter* strains were Gm1/gene 3.21 positive but only 5% of community isolates of *Campylobacter* were Gm1/gene 3.21 positive, this suggests that both are required for neuropathogenicity. To test this hypothesis, gene 3.21 negative mutants could be produced from a strain of GBS-associated *C. jejuni* by a suitable mutagenesis system. Animal model studies with the 3.21 negative mutant and wild type strain of GBS-associated *C. jejuni* would then be performed. Data collection regarding nerve damage would then provide valuable information into the dependant relationship of these two genes.

Outer membrane characteristics are often important virulence determinants in bacteria. While the genome of *C. jejuni* has recently been sequenced, only a few definite protein antigens have so far been characterized. For example, serological studies have shown that the 65-kDa flagellin, the 44-kDa surface protein and the 25- to 29-kDa surface proteins all react to the convalescent sera of patients with *Campylobacter* gastrointestinal infections (Blaser, Hopkins and Vasil, 1984; Nachamkin and Hart, 1985; Wenman *et al*, 1985; Dunn, Blaser and Snyder, 1987). Also, protein PEB1 is thought to function as an adhesion for attachment to eukaryotic cells (Pei and Blaser, 1993) and the heat shock protein GroEL elicits an immune response in infected rabbits (Wu *et al*, 1994) as does the *Campylobacter* trigger factor in humans (Griffiths, Park and Connerton, 1995). These studies have characterized common proteins found in gastrointestinal isolates of *Campylobacter*. This is the first study to report a GBS-specific "probable" membrane protein which is found in a high proportion of GBS-associated control strains of *C. jejuni* whilst being relatively uncommon in community isolates from patients with gastrointestinal infections.

6.6: Clone 3.21 gene expression experiments

6.6.1 Method

To determine if the probable membrane protein that the gene associated with clone 3.21 codes for could be detected by SDS-polyacylimide gel electrophoresis (SDS-PAGE), a number of different experiments were performed. Firstly, SDS-PAGE of whole cell protein extracts was performed on two related *C. jejuni* isolates (as determined by PFGE), a clone 3.21 positive *C. jejuni* and a clone 3.21 negative *C. jejuni*. Secondly, SDS-PAGE was performed on *E. coli* transformed with the pT-Adv vector containing the 3.21 DNA insert identified from the subtracted library. This was compared to the same strain of *E. coli* with a non-recombinant plasmid. Thirdly, as the open reading frame of gene 3.21 was unknown, three PCRs were developed to amplify clone 3.21DNA using three forward primers of varying length (Veh18, Veh19 and Veh20) and the same reverse primer (VehRev) (Figure 6.11). The optimised reaction mixture for PCR and the thermal cycling conditions using the Perkin Elmer DNA

Figure 6.11: Primer design for gene 3.21 cloning experiment

GCAATNCTATCATCAAAATGCAAATTTCTTTTAATAATGAAAGCA

AAGAAATTTTACTTTTTNCAAATTACAACAATGAAAATGTTTTAC
CTTTTAGATTAGGAGGTGAAATTTTTGCACTCAACTGGGGTCCTG
AAGAGATTAAACTGCCTTTTTCTTTGGCTTTAAAAGATTTTATCCT
TGATCGCTACGCAGGTTCTATGAGTCCTTCTTCTTATGCTTCAGAT
ATCGAAGTGATCGATCAAGATAAAAGTTTTGAGTATAAAATTTTT
ATGAATAATGTTTTAGATTATGGCGGATATAGATTTTTTCAAAGT
TCTTATGATCAAGACGAACAAGGCACCATACTTTCAGTCAATAAA
GATCCTGGAAAAATTCCAACTTATATAGGCTATACTTTGCTGACT
TTAGGTTTTTTATGGGATTTTATTTGCTAAAAACTCAAGATTTCAA
AAATTATCAAACTATCTTAAAAATCAAAAAAATCTTTTATTAATT
CTTTTTTGNCTTTTTGCTTTTAACATAAAAAAGGTTTTGCCCGATG
AAAACACCTTAAAGACCTC

Veh 18 Forward primer	
Veh 19 Forward primer	
Veh 20 Forward primer	
VehRev Reverse primer	

Thermal Cycler Model 480 were the same as given in Tables 6.1 and 6.2 respectively with the following exceptions. The annealing temperature was lowered to 50°C and the Magnesium Chloride concentration altered to 3.3 mM. The subsequent products of 569 b.p, 570 b.p and 571 b.p in size were ligated into the expression vector, pQE 30 UA (QIAGEN) and transformed into TOP10F' *Escherichia coli* cells and plated onto LB/X-gal/IPTG agar plates containing 50 μg/mL of kanamycin. SDS-PAGE was performed on white colonies containing DNA of clone 3.21 and compared to *E. coli* containing the same expression vector with no extra inserted DNA.

6.6.2 Results

All three experiments showed no apparent differences in protein profiles compared to their relative controls (Figure 6.12).

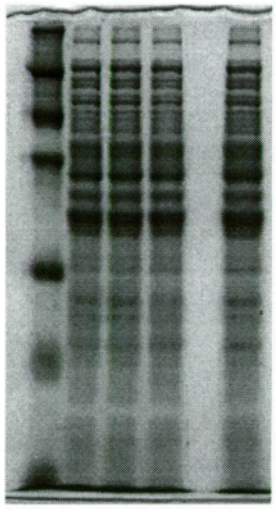
6.7 Summary

Subtractive hybridisation has been successfully used to identify a DNA region that is found in the majority of GBS strains tested and that is relatively uncommon in community gastrointestinal isolates of *Campylobacter.* As only a small number of GBS strains were tested in this study, screening of larger GBS *Campylobacter* culture collections is needed to determine the true incidence of the gene across other serotypes that have been associated with GBS, however for reasons referred to previously, *Campylobacter* isolates from GBS patients are rare.

A PCR method based upon this gene has been developed that allows for rapid identification of those strains that are potentially neuropathogenic.

Preliminary protein analysis has so far provided no information about the nature of the "probable" membrane protein associated with this gene. Further sequence information must be obtained upstream and downstream of the known DNA sequence described here in order to determine the entire coding region for the gene so that expression experiments can be repeated. Furthermore, a high efficiency mutagenesis system could be developed to produce gene knock-out mutants allowing for identification and characterization of expressed proteins in the wild-type strain but not the mutants. Both of these experiments are outside the scope of this study.

Figure 6.12: SDS-PAGE analysis of whole cell extracts of *E. coli* containing expression vector containing clone 3.21 DNA



Lane 1 2 3 4 5

Lane 1: Molecular Weight Marker, Lanes 2-4: *E. coli* containing clone 3.21 DNA/Expression vector, Lane 5: Control *E. coli* with expression vector only

CHAPTER 7

7.1 CONCLUSIONS

The first reported case of *Campylobacter*-associated GBS was published by Rhodes and Tattersfield (1982). As GBS often presents many weeks following a gastrointestinal infection with *Campylobacter* making isolation of the bacterium from faeces impossible in the majority of cases, the link between infection and GBS has been made by serological studies. Over the last 20 years, with improved culture techniques, more isolates have been collected and today, study groups around the world have large, characterised collections of *C. jejuni* strains associated with GBS.

The reasons why some patients develop GBS and others do not is still unknown. Some researchers have hypothesised that individual patient susceptibility is the key factor predisposing them following infection with this organism. Others have theorised that the organisms themselves have some unique neuropathogenic characteristic. Studies of the bacterium have shown that cell wall lipopolysaccharides can mimic human neuronal ganglioside structures such as Gm1. Antibodies produced following gastrointestinal infection with *Campylobacter* are therefore thought to lead to autoimmune responses to the patient's neurones resulting in this neurological symptom. While many serotypes of *Campylobacter* possess these cell surface molecules, certain serotypes, in particular Penner serotype O:19, appear to be over represented in most studies of GBS. Other serotypes isolated from GBS patients include O:1, O:2, O:4, O:4 complex, O:5, O:10, O:16, O:23, O:37, O:41, O:44 and O:64.

Recent epidemiological studies have been applied to characterising this group of organisms. Analysis of GBS and MFS strains by AFLP, PFGE, *fla*A polymorphism and randomly amplified polymorphic DNA (RAPD) analysis have not shown a unique type that can differentiate GBS-associated strains from non-GBS/MFS strains.

In the present study, PFGE was shown to be an effective epidemiological technique allowing for the differentiation of gastrointestinal *Campylobacter* isolates for the identification of outbreak strains. Furthermore, cluster analysis of PFGE profiles of *C. jejuni* and *C. coli* by available computer software allowed for identification to species level. Only a limited range of other species were studied, however, and as such, the use of PFGE to differentiate them cannot be assessed. As was the case in the study by Endtz *et al*, (2000), PFGE could not be used to differentiate GBS strains from non-GBS strains. While there was close homology with serotype O:19 GBS isolates by this technique, all other serotyped GBS isolates had variable profiles. Cluster analysis of PFGE profiles did however differentiate overseas GBS strains as a group from

Tasmanian community isolates of *Campylobacter.* As the one Tasmanian GBS strain in our study clustered with the Tasmanian community strains and not with the GBS strains, it might be concluded that this was due to a geographical phenomenon rather than a characteristic of GBS strains.

Characterisation of Tasmanian community *Campylobacter* isolates to determine the presence of Gm1epitope and the prevalence of serotype O:19 have also been performed as part of this study. The rate of Gm1 positive *Campylobacter* strains seen in Tasmania was 37.2 per 100,000 population over the time period studied. As the GBS rate in Tasmania over the same period was 2.3 per 100,000 population, it can be concluded that factors other than the presence of Gm1-like epitopes in the cell wall of *Campylobacter* species are needed for development of GBS. Furthermore, while serotype O:19 is not the only serotype associated with GBS, due to its high prevalence in previous GBS studies, it was important to determine the prevalence of this particular serotype in the Tasmanian community. As such, a previously published PCR assay for the serotype O:19 specific gyrase B gene (Misawa , Allos and Blaser, 1998) was used to investigate O:19 incidence in the Tasmanian population. No isolates in our study were found to belong to this serotype. This result was surprising as a study from USA found that 2% of patients with uncomplicated gastroenteritis were infected with the serotype O:19 strain (Mishu, Patton and Blaser, 1993). Furthermore, in this study some serotype O:18 and non-typable strains produced false positives using this assay in 3.2% of isolates tested.

C. jejuni has been the predominant *Campylobacter* species isolated in association with GBS, but a recent isolation of *C. upsaliensis* has been reported (Ho *et al*, 1997). Speciation of isolates was therefore important to characterise other species that may be potentially neuropathogenic.

As this group of organisms is biochemically inert and difficult to identify, a molecular based method was developed in our study to rapidly identify *Campylobacter* isolates. A multiplex PCR was developed to detect both genus specific 16S rRNA for *Campylobacter* and *Arcobacter* species as well as the hippurate gene for *C. jejuni.* In our study, 237 of 250 isolates (94.8%) were identified as *C. jejuni* within 4 hours, including 2 isolates that were biochemically hippurate negative. The remaining 13 isolates were identified as belonging to the *Campylobacter/Arcobacter* group with positive 16S rRNA PCR detection. This PCR product was subsequently used in a dot blot hybridisation assay with species specific biotinylated probes to *C. coli, C. upsaliensis, C. fetus, C. lari* and a genus specific probe to *Arcobacter.* Using this system, 10 isolates were identified as *C. coli,* 2 as *C. lari* and 1 *C. upsaliensis.* As isolation of non-*C. jejuni Campylobacter* are infrequent, their neuropathic nature is hard to assess. Future improvements to culturing techniques and/or PCR detection methods from faecal samples may

provide more accurate data regarding the prevalence of other *Campylobacter* species within the community and potential associations with GBS.

Serotyping, Gm1 analysis and PFGE provide useful information about the nature of GBS associated strains of *Campylobacter* but lack the ability to discriminate between those that only cause gastroenteritis and those that may invoke GBS. A closer study of GBS-related strains at the genomic level may provide information on unique characteristics of these strains and subsequently lead to development of detection methods to quickly identify these strains allowing prompt medical intervention.

The comparative genomic method, subtractive hybridisation, is a powerful technique for the isolation of genes present in one bacterial cell population but absent in another. It was used to compare a known strain of *C. jejuni* associated with GBS (serotype O:19) with a strain from a serotype that has not been associated with GBS (serotype O:3). Unique regions of DNA were cloned into *Escherichia coli* and analysed by hybridisation to digoxigenin labeled DNA from a GBS *C. jejuni* strain to remove non-specific fragments. After testing one hundred such clones, one positive clone which contained the DNA fragment (*Veh*) was identified which hybridised to DNA from 11 GBS control isolates (representing 5 strains) and 14.5% of gastroenteritis strains of *Campylobacter*.

Following sequence analysis of this *Veh* fragment, primers were designed and a PCR-based method developed to rapidly detect the *Veh* gene. Utilizing this assay, the *Veh* gene was detected in 91% of control GBS strains of *C. jejuni* and 10.5% of community isolates of *Campylobacter*. This method detected fewer positives than the crude *Veh* gene probe used previously due to the specific nature of the designed PCR primers. Importantly, community isolates that were detected by this method, comprised both Gm1 positive and Gm1 negative strains, indicating that the unique *Veh* region detected was not related to the genes coding for Gm1-like epitopes found in the lipopolysaccharides of some isolates.

Genbank DNA matches of the sequenced DNA showed that the highest level of homology was found to be to a 138 nucleotide sequence from *C. jejuni* subsp *jejuni* NCTC 11168. This region corresponds to designated gene "Cj1013c", a probable membrane protein of undefined function as determined by the *C. jejuni* sequencing data held at the Wellcome Trust Sanger Institute, Cambridge, UK. This represents the first non-ganglioside based marker of GBS-associated *Campylobacter* reported to date.

7.2 Further work

It is hypothesised that the *Veh* gene may be important in the pathogenesis of campylobacters that

are associated with GBS. While current evidence suggests the gene codes for a membrane protein, we still have limited knowledge of the function of this gene product and therefore a suitable, high efficiency mutagenesis system for *C. jejuni* will need to be developed for further research. Once optimized, the mutagenesis system could be used to produce *Veh* isogenic mutants which could then be used for proteome analysis using two dimensional SDS-PAGE and high-sensitivity mass spectrometry. Comparison of these mutant strains deficient in the *Veh* gene with the wild type strain would provide valuable information into the nature of the unknown protein.

An important future development to aid the study of *Campylobacter*-associated GBS would be the development of a good animal model, something that has not been available to researchers to date. This would allow challenge studies of *Veh* immunised animals to be performed.

While a rapid 4 hour PCR method has been developed in the present study to detect isolates of *Campylobacter* possessing the *Veh* gene, purification of the *Veh* protein and production of antisera to various epitopes of the *Veh* gene product would allow the development an antigen detection system. Production of a rapid latex agglutination test for laboratory staff to identify neuropathogenic strains within 10 minutes of isolation would then be possible.

If the hypothesis that the *Veh* gene is important in the pathogenesis of *C. jejuni*-associated GBS is proved to be correct, then it would become possible to direct the development of prophylactic and therapeutic interventions for *C. jejuni*-associated GBS.

APPENDIX A: Phenotypic properties of *Campylobacter* species

Organism	Catalase	Nitrate reduction	Nitrite reduction	H_2 required	Urease	H_2S (TSI)	Hippurate hydrolysis	Indoxyl acetate hydrolysis
C. jejuni subsp. *jejuni*	+	+	-	-	-	-	+	+
C. jejuni subsp. *doylei*	V	-	-	-	-	-	V	+
C. coli	+	+	-	-	-	-	-	+
C. fetus	+	+	-	-	-	-	-	-
C. fetus subsp.*venerealis*	+	+	-	-	-	-	-	-
C. lari	+	+	-	-	V	-	-	-
C. upsaliensis	W	+	-	-	-	-	-	+
C. hyointestinalis	+	+	-	V	-	+	-	-
C. sputorum biovar *sputorum*	-	+	+	-	-	+	-	-
C. sputorum biovar *bubulus*	-	+	+	-	-	+	-	-
C. sputorum biovar *fecalis*	+	+	+	-	-	+	-	-
C. helveticus	-	+	ND	-	ND	-	-	+
C. mucosalis	-	+	+	+	-	+	-	-
C. concisus	-	+	+	+	-	+	-	-
C. curvus	-	+	+	+	-	+	-	+
C. rectus	-	+	+	+	-	+	-	+
C. showae	+	+	ND	+	-	+	-	+
A. cryaerophilus group 1A	+	V	-	-	-	-	-	+
A. cryaerophilus group 1B	+	V	ND	-	-	-	-	+
A. butzleri	W	+	-	-	-	-	-	+
A. nitrofigilis	+	+	-	-	-	-	-	-
A. skirrowii	+	+	ND	-	-	-	-	+

TSI, triple sugar iron; W, weak reaction; V, variable reaction; ND, not determined (Nachamkin, 1999)

Organism	Growth at 15°C	Growth at 25°C	Growth at 42°C	Growth in 3.5% NaCl	Growth in 1%Glycine	Growth on MCA	Susceptiblity to Nalidixic acid	Susceptibility to Cephalothin
C. jejuni subsp. *jejuni*	-	-	+	-	+	+	V	R
C. jejuni subsp. *doylei*	-	-	-	-	+	-	S	S
C. coli	-	-	+	-	+	+	S	R
C. fetus	-	+	-	-	+	+	V	S
C. fetus subsp. *venerealis*	-	+	-	-	–	+	R	S
C. lari	-	-	+	-	+	+	R	R
C. upsaliensis	-	-	+	-	V	-	S	S
C. hyointestinalis	-	+	+	-	+	+	R	S
C. sputorum biovar *sputorum*	-	-	+	-	+	+	S	S
C. sputorum biovar *bubulus*	-	-	+	+	+	-	R	S
C. sputorum biovar *fecalis*	-	-	+	-	+	+	R	S
C. helveticus		-	+	V	V	ND	S	S
C. mucosalis	-	-	+	-	+	+	R	S
C. concisus	-	-	+	-	+	+	R	R
C. curvus	-	-	+	-	+	ND	S	ND
C. rectus	-	-	W	-	+	ND	S	ND
C. showae	-	-	+	-	V	ND	R	S
A. cryaerophilus group 1A	+	+	-	-	-	-	V	R
A. cryaerophilus group 1B	+	+	-	-	-	+	S	V
A. butzleri	+	+	V	V	+	+	S	R
A. nitrofigilis	+	+	-	+	-	-	S	S
A. skirrowii	+	+	V	V	V	-	S	S

MCA, MacConkey Agar; W, weak reaction; V, variable reaction; ND, not determined; S, susceptible; R, resistant (Nachamkin, 1999)

APPENDIX B: Preparation of LB (Luria-Bertani) medium (pH 7.0) with antibiotics. Taken from Clontech AdvanTAge™ PCR Cloning Kit User Manual.

LB (Luria-Bertani) medium (pH 7.0)

		for 1 L:
1.0%	Bacto-tryptone	10 g
0.5%	Yeast extract	5 g
1.0%	NaCl	10 g

Dissolve ingredients in 950 ml of deionized water. Adjust the pH to 7.0 with 5 M NaOH and bring the volume up to 1 L. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Store at room temperature or at 4°C.

LB/antibiotic plates

Prepare LB medium as above, but add 15 g/L agar before autoclaving. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Let cool to ~ 55°C, add antibiotic (50 µg/mL of either ampicillin or kanamycin), and pour into 10 cm plates. Let harden, then invert and store at 4°C.

APPENDIX C: Reagents and solutions used for the dot blot hybridisation identification assay. Taken from "The DIG System User's Guide for Filter Hybridisation manual (Boehringer Mannheim)

Solution	Constituents
Prehybridisation solution	5 X SSC, 0.1% sodium-lauroylsarcosine, 0.02% SDS, 1% blocking solution
Blocking solution (10%)	150 mM NaCl, 100 mM maleic acid, pH 7.5, 10% (w/v) Blocking Reagent
Washing solution 1	2 X SSC, 0.1% SDS
Washing solution 2	0.5X SSC, 0.1% SDS
Washing solution 3	150 mM NaCl, 100 mM maleic acid, pH 7.5
Detection solution	100 mM Tris-HCl, 100 mM NaCl pH 9.5
20X SSC solution	3 M NaCl, 0.3 M sodium citrate, pH 7.0

APPENDIX D: Reagents and solutions used for Pulsed-Field Gel Electrophoresis Smith and Cantor, 1987.

Solution	Constituents
PIV solution	10 mM Tris-Cl (pH7.6), 1M NaCl
ESP solution	0.5M EDTA (pH9.5), 1mg/ml Proteinase K, 1% N-laurylsarcosine, sodium salt
TE1 solution	10 mM Tris-Cl (pH7.6), 1 mM EDTA (pH8.0)
5 X TBE solution	0.445M Tris Base, 0.445M Boric acid, 10 mM EDTA (pH 8.0)
Restriction buffer A	33 mM Tris-acetate, 66 mM Potassium-acetate 10 mM Magnessium-acetate, 0.5 mM dithioerythritol (pH 7.9)
Restriction buffer H	50 mM Tris-HCl, 100 mM Sodium Chloride, 10 mM Magnessium Chloride, 1 mM dithioerythritol, (pH 7.5)

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